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Vol. 13, No. 1 *Contents* *August 1955*

JEBB, W. H. H. and TOMLINSON, A. H. The nutritional requirements of *Haemophilus pertussis*.
MAYR-HARTING, ANNA. The acquisition of penicillin resistance by *Staphylococcus aureus*, strain H/Oxford.
COOPER, P. D. The site of action of penicillin: some changes in *Staphylococcus aureus* during the first two hours' growth in penicillin media.
MILLS, R. F. N. The action of fumagillin on a bacteriophage of *Staphylococcus aureus*.
BIGGER-GEHRING, LOIS. Transamination reactions in *Saccharomyces fragilis*.
POWELL, JOAN F. and HUNTER, J. R. The sporulation of *Bacillus sphaericus* stimulated by association with other bacteria: an effect of carbon dioxide.
POWELL, JOAN F. and HUNTER, J. R. Spore germination in the genus *Bacillus*: the modification of germination requirements as a result of pre-heating.
DAVIS, G. H. G., BISSET, K. A. and HALE, C. M. F. Correlation between morphological and physiological characters in the classification of members of the genus *Lactobacillus*.
MATHIESON, M. JEAN and CATCHESIDE, D. G. Inhibition of histidine uptake in *Neurospora crassa*.
DICKENSON, P. B. and MACDONALD, K. D. An electron microscope examination of the initial cell stage in *Streptomyces* spp.
KLECZKOWSKI, A. The statistical analysis of plant virus assays: a transformation to include lesion numbers with small means.
BUXTON, E. W. and RICHARDS, M. G. Pathogenic strains of *Fusarium oxysporum* Fr. distinguished by their differential tolerance to inhibition by various actinomycetes.
HARRIS, W. J. Demonstration of the existence of spore sacs around resting spores of *Bacillus subtilis* and *Bacillus globigii*.
TOMCSIK, J. and GRACE, JOYCE B. Bacterial cell walls as revealed by the specific cell wall reaction and by direct staining with Alcian Blue.
SPENCER, R. The taxonomy of certain luminous bacteria.
ERIKSON, DAGNY. Changes in refractility and permeability accompanying germination of heat-resistant spores of *Micromonospora vulgaris*.
ERIKSON, DAGNY. Thermoduric properties of *Nocardia sebivorans* and other pathogenic aerobic actinomycetes.
ERIKSON, DAGNY. Loss of aerial mycelium and other changes in streptomycete development due to physical variations of cultural conditions.
CUCKOW, F. W. and KLIENEBERGER-NOBEL, E. Further studies of organisms of the pleuropneumonia group by electron microscopy.
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CONTENTS OF THE APRIL ISSUE

General Meeting.

The Solids-not-Fats Problem, by Dr A. L. PROVAN.

The Economics of Retail Milk Distribution—Present and Future Trends, by L. S. WHEELER, A.C.A.

British Standards.

Retail Distribution, by C. S. KIRBY.

Dairying History No. 7: A History of Laboratory Control, by E. B. ANDERSON.

Book Review.

Detergency, by F. H. PERRIN.

The Cost of Washing and Sterilizing Milking Equipment on Farms, by D. N. AKAM.

Personalia.

The Effect of Penicillin in Milk Used for the Manufacture of Cheshire Cheese, by S. B. THOMAS, J. J. PANES and JOHN LEWIS.

Sectional Notes.

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Editor: K. C. Sen, D.Sc., F.N.I.

CONTENTS OF VOL. VII, No. 2, JUNE 1954

K. C. Sen, B. N. Premachandra, G. K. Murthy, Noshir N. Dastur and D. Narayan. The Effect of Feeding Iodinated Casein on Yield and Composition of Milk.—I. Sirry and S. D. Rakshy. Pickling of Ewes Milk Danni *Cheese.—G. S. Mani, M. R. Srinivasan and S. C. Ray. Studies on the Production of Ghee (Clarified Butterfat) Directly from Cream. Part 2. The Qualitative Factors in Cream Affecting Its Washability for Reduction of Serum-Solids.—K. M. Narayanan, N. B. Shroff, C. P. Anantakrishnan and K. C. Sen. Vitamin A in Dairy Products. Part 2. Vitamin A in Curd and Ghee from Fortified Milk.—H. Laxminarayana and K. K. Iya. Tetrazolium Reduction Test for Quality Control of Milk.—G. K. Murthy, Noshir N. Dastur and S. C. Ray. Effect of Feeding Iodinated Casein on Calcium and Phosphorus Metabolism in Young Heifers.—J. Babad, A. Pinsky and N. Sharon. Calculation of Total Solids in Milk of Cows in Israel by Richmond's Formula.—M. M. Taha El-Katib. Relation of Stage of Lactation to Volatile and Unsaturated Fatty Acids of Egyptian Cow and Buffalo Butterfat.—Reviews.*

CONTENTS OF VOL. VII, No. 3, SEPTEMBER 1954

S. De and S. C. Ray. Studies on the Indigenous Method of Chhana-*Making. Part I. The Influence of the Conditions of Coagulation and the Type of Milk on the Production of* Chhana.*—S. Balakrishnan and R. Rajagopalan. Effect of Feeding Curds, Sulphaguanidine and* Para-*Amino Benzoic Acid on the Coliform Organisms and Biosynthesis of Thiamine.—A. A. AskerM. T. Ragab and (Miss) S. A. Hilmy. Longevity in Egyptian Cattle and Buffaloes.—N. V. Joshi and H. Raj. Microbiological Assay of Essential Amino Acids in the Milk of Indian Buffalo.—T. Narayanan and K. K. Iya. Studies on Bovine Mastitis. II. Methods of Diagnosis.—N. B. Shroff, S. R. Sampath, C. P. Anantakrishnan and K. C. Sen. Vitamin A in Dairy Products. Part III. Heat Stability of Vitamin A in Ghee.*

VOLUME 22, NO. 2 JUNE 1955

636.2.082.4

579. A STUDY OF VARIATION IN TWIN CATTLE

IV. EMERGENCE OF PERMANENT INCISOR TEETH

BY G. WIENER AND H. P. DONALD

Agricultural Research Council Animal Breeding Research Organization, Edinburgh

Permanent incisor teeth of cattle are commonly used for estimating age, but there is little information about either the magnitude or sources of normal variation in age at eruption. An opportunity to study this variation was presented by the uniformity trial of twins and half-sibs discussed in earlier papers in this series and described in the first (4). The data also furnish a useful test of the possibility suggested by King & Donald (7) that the variation shown within pairs of one-egg twins might not be a correct measure of environmental effects within pairs of half-sisters treated uniformly like the twins. If increasing departure from genetic identity attracts increasing contributions of variation not due to the additive effects of genes, the point is of considerable practical and theoretical importance. The purpose of this paper, therefore, is to examine this point as well as some of the sources of the variation in age at eruption of incisor teeth shown by liberally fed cattle.

Youatt (19) gave 2, 3, 4 and 5 years as the approximate ages at which to expect the 1st, 2nd, 3rd and 4th pairs of permanent incisors. In a most detailed account, Simonds (12) stated that age at eruption is influenced by breeds and 'other causes'. He divided cattle into early and late groups on the basis of differences in this character. According to Miller & Robertson (9) ages at eruption are influenced by 'domestication, methods of management and the nature of food, and what applies to the more highly specialized improved breeds does not apply to the commonly bred cattle, and what applies to these latter does not apply to ranch cattle'. Zorn (20) is also of the opinion that breed and feeding, as well as individuality, account for variation in age at eruption of incisors in German cattle. Brown (3), however, in a booklet on dentition, written originally at the beginning of this century but recently revised, questions the truth of the assumption that breeding and feeding affect the emergence of teeth. He states that over more than half a century, during which the management of cattle altered appreciably, he noted no marked changes. Average ages at eruption are quoted by many other authors, but the source and extent of their observations are not always apparent.

The regulations of the Smithfield Club Show (14) in respect of permanent incisor teeth are: 'Cattle having their central permanent incisors cut will be considered as exceeding one year and six months. Cattle having their central permanent incisors fully up will be considered as exceeding one year and nine months. Cattle having their second pair of permanent incisors fully up will be considered as exceeding two years and three months. Cattle having their third pair of permanent incisors cut will be considered as exceeding two years and eight months. Cattle having their fourth pair (corner) permanent incisors fully up and their anterior molars showing signs of wear will be considered as exceeding three years.' Most authors report slightly greater average ages. The Smithfield ages might

be chosen therefore either to make allowance for the relatively early eruption of teeth claimed for high quality beef cattle, or to establish a workable definition of age.

Franklin (5), and workers quoted by him, have shown that food deficient in calcium will adversely affect both incisor and molar development in sheep. Tooth size in mice was particularly sensitive to the kind of food their dams were given during pregnancy and lactation (11). Thyroidectomized sheep showed delayed eruption of teeth, though the order of eruption was not changed (8, 16). In man a number of conditions are known to delay eruption, but very few which speed its onset (15). It may be concluded that certain gross dietary or physiological deficiencies will cause deviations from normal eruption and growth of teeth, but that not much is known of the sensitivity of developing teeth to the less conspicuous influences of heredity and husbandry.

MATERIAL AND METHODS

For the present study, fifteen pairs of one-egg twins (MZ), fourteen pairs of two-egg twins (DZ), and thirteen pairs of half-sibs (HZ) were available. All the animals, for the care of which Mr D. Anderson was responsible, were on the same high plane of nutrition throughout their life and belonged to a variety of breed types and crosses. Fortnightly observations, limited to permanent incisors, were made from the age of 15 months. Five stages of eruption were recognized and recorded as 1 (just through the gum), 2, 3, 4 and 5 (fully up). Complete emergence was, like other stages in eruption, judged subjectively. Owing to gradual culling of the animals after their first lactation, the number of observations available for analysis diminished with each successive pair of teeth.

It should be added that what is described here as the 4th pair of permanent incisors is regarded by dental anatomists (17) as a pair of mandibular canines. The use of the term 'incisor' for this pair is based on the nomenclature of Sisson & Grossman (13).

RESULTS

Age at eruption of permanent incisors

Age at eruption is highly variable—more so than might be supposed from the practical use to which it is put. First incisor teeth may emerge as much as 5 months before or after the due date (about 2 years) and later teeth vary even more. The result is that some animals have, for instance, their 3rd pair at an earlier age than that at which others have their second pair. As shown in Table 1, which records the age at eruption for each tooth individually, the earliest of the 1st incisors in this material appeared soon after 18 months of age and the last of the 4th incisors at 4 years and 10 weeks. The distributions of ages at eruption show a slight positive skewness for all pairs of teeth, which increases from 1st to 2nd to 3rd incisors; that for the 3rd incisors is statistically significant ($P = 0{\cdot}02$). Skewness for the 4th incisor is somewhat less than for the 3rd on one test for skewness and somewhat greater on another.

The increasing range of ages noted in Table 1 is associated with an increasing variance in age at eruption (Table 2) such that the coefficient of variability remains constant at about 8%.

The average interval between eruptions of successive pairs was roughly 6 months, though possibly the last pair was a little overdue on this reckoning. Means and variances

Table 1. *Distribution of age at eruption of permanent incisor teeth*

Age at eruption (weeks)	1st	2nd	3rd	4th
79–82	1	—	—	—
83–86	4	—	—	—
87–90	15	—	—	—
91–94	27	—	—	—
95–98	26	—	—	—
99–102*	32	—	—	—
103–106	31	—	—	—
107–110	14	—	—	—
111–114	12	11	—	—
115–118	4	17	1	—
119–122	2	24	—	—
123–126	—	24	—	—
127–130*	—	23	4	—
131–134	—	24	6	—
135–138	—	11	7	—
139–142	—	7	15	—
143–146	—	6	13	—
147–150	—	1	25	—
151–154*	—	—	21	2
155–158	—	—	6	3
159–162	—	—	8	9
163–166	—	—	6	2
167–170	—	—	5	11
171–174	—	—	4	13
175–178	—	—	4	9
179–182*	—	—	1	8
183–186	—	—	1	8
187–190	—	—	1	7
191–194	—	—	—	9
195–198	—	—	—	6
199–202	—	—	—	2
203–206	—	—	—	4
207–210	—	—	—	2
211–214	—	—	—	—
215–218	—	—	—	1
Totals	168	148	128	96

* Mean value included in range.

Table 2. *Age at eruption of pairs of permanent incisors in cattle twins and half-sibs*

Incisor pair	No. of animals	Mean age at eruption (weeks)	Mean square of age at eruption	Mean interval between eruptions (weeks)
1st	84	100·4	58·2	—
2nd	74	127·1	67·8	26·7
3rd	64	151·2	153·8	24·1
4th	48	180·0	199·4	28·8

in Table 2 apply approximately to the three contributing groups, and to the forty-eight animals from which data are complete.

Although ages at eruption have been averaged for each pair of teeth in Table 2, there is some variation between left and right teeth which requires notice. In 30% of the 270 pairs of teeth studied the left and right tooth of each pair erupted during the same fortnight; the left tooth appeared first by one or more observation periods in another 30%; and in 40% of all pairs the right tooth erupted before the left. Furthermore, when the right erupted before the left member of a pair, the time interval between the two appearances was found to be a little greater than in pairs of which the left came first. The net effect, averaged over all animals and pairs of teeth, was that right teeth appeared nearly 10 days

before left teeth—a difference which is statistically highly significant. The interval, however, between eruption and full size of each tooth was about 2 days shorter for left than for right members of each pair of teeth. This also is statistically significant in aggregate. The compensatory effect of quicker growth after later eruption of left teeth resulted in their reaching full size about a week after right teeth.

As a measure of symmetry, the age at eruption of the left tooth was subtracted from that of the right. To determine whether heredity governed this expression of symmetry, the 'right-left' value for one member of each twin pair was subtracted from the 'right-left' value obtained from the other member. The within-pair variance was then calculated from the differences in these values. This variance from MZ twins was only about half that obtained from the DZ twins for the 1st and 2nd incisors. For 3rd and 4th incisors the variance from MZ twins was slightly greater than that from DZ. Within-pair variances from HZ were less than those from DZ pairs for 1st and 2nd incisors but appreciably larger for 3rd and 4th incisors.

The observations would be consistent with some degree of genetic control. There might be in addition some 'right-left' asymmetry in individuals due to observational bias or to the direction of movement of the lower jaw in chewing.

Sources of variation in age at eruption

Although the total variation in each of the three groups of animals was much the same, the three fractions into which it has been divided for each group are neither equal nor consistent. In terms of mean squares, the within-animal variation (that is, variation within a given pair of teeth) is relatively small and similar for all groups. It tended to increase slightly from 1st to 4th incisors. This agreement of the three groups is to be expected but ceases at this point. Whereas one member of an MZ pair agreed with the other as well as one tooth did with its mate, DZ twins on average differed noticeably for all teeth. HZ pairs showed still more intra-pair variation; as they should on genetic grounds. What the MZ twins lacked in variety within pairs they more than made up in differences between pairs. In this respect they exceeded both DZ and HZ pairs—a distinction they may have owed to an original genetic sampling diversity. Of these generalizations, which are based on the facts given in Table 3, the most interesting relates to the apparent failure of any accidents of environment to make MZ twins any less alike than right and left sides of the same animal. Such accidents seem to have been of no importance at all in MZ twins nor, by inference, in DZ and HZ pairs.

Heritabilities may be calculated from the within-pair variances in Table 3 on assumptions set out by Donald (4) and modified by King & Donald in the paper on growth (7). Briefly, variation arising within MZ pairs is thought of as entirely environmental (e'^2); variation arising within DZ pairs contains the whole of e'^2 and half the additive genetic variance ($\frac{1}{2}g'^2$), and that within HZ pairs the environmental component (e'^2), three-quarters of the additive genetic variation ($\frac{3}{4}g'^2$), and a component m'^2 containing variance not otherwise accounted for. Heritability (h^2) is then taken to be the ratio of the genetic variance (g'^2) to the total variance ($g'^2+e'^2+m'^2$). The argument can be simplified by assuming that the variance arising within HZ pairs contains only $e'^2+\frac{3}{4}g'^2$; the e'^2 from MZ twins is then assumed to be applicable to half-sibs. This, however, may be too risky an assumption to make. The HZ variance has, therefore, been

regarded as containing an m'^2 component which has been isolated by deducting estimates of e'^2 and $\frac{3}{4}g'^2$ obtained from the twin data. Table 4 in line (*a*) gives estimates of heritability where e^2_{HZ} is assumed to differ from e^2_{MZ} and e^2_{DZ} by this component m'^2. These estimates are markedly different from those in lines (*b*) and (*c*) of Table 4 where the presumption holds that $m'^2=0$. Line (*b*) is derived from twins only. Line (*c*) shows the results of assuming $m'^2=0$ and using MZ, DZ and HZ intra-pair variances to yield two separate estimates for both e'^2 and g'^2. These have been combined by the least squares procedures into single estimates from which the ratio h^2 has been derived. The intra-class correlation of MZ pairs is sometimes used as an estimate of heritability (line (*d*)) on the assumption that environmental variation in one-egg twins is the full measure of this variation in unrelated animals.

Table 3. *Analysis of variance of age at eruption of permanent incisors in twins and half-sibs*

	Incisors							
	1st		2nd		3rd		4th	
Source	DF	MS	DF	MS	DF	MS	DF	MS
	One-egg twins (MZ)							
Between pairs	14	73·9	12	77·0	10	195·6	7	303·3
Within pairs	15	1·3	13	1·1	11	1·7	8	3·7
Within animals	30	0·8	26	1·1	22	3·1	16	1·8
	Two-egg twins (DZ)							
Between pairs	13	40·7	11	70·8	9	100·0	6	91·8
Within pairs	14	12·4	12	10·8	10	32·5	7	29·3
Within animals	28	1·5	24	2·8	20	1·7	14	2·8
	Half-sibs (HZ)							
Between pairs	12	41·2	11	30·9	10	66·6	8	79·9
Within pairs	13	11·6	12	18·1	11	86·8	9	123·5
Within animals	26	1·0	24	1·4	22	4·8	18	4·5

Table 4. *Heritabilities of age at eruption of incisors*

	Incisors			
h^2 calculated from:	1st	2nd	3rd	4th
(*a*) MZ, DZ, HZ	1·29	0·85	0·60	0·38
(*b*) MZ and DZ only	0·94	0·95	0·97	0·93
(*c*) Least squares method (MZ, DZ, HZ)	0·90	0·95	0·98	0·95
(*d*) Intra-class correlation of MZ	0·96	0·97	0·98	0·97

The heritabilities in Table 4 are likely to be slight overestimates to the extent that variance due to dominance, epistasis and genotype-environment interactions are included in g'^2 (7). However, the estimates in lines (*b*), (*c*) and (*d*) are so high that, even assuming some error due to these causes, there appears little scope for non-genetic variation. These possible errors will have relatively little effect on the age trend in h^2 in line (*a*); this will be further discussed below.

Correlation between age at eruption of successive teeth

Eruption of successive teeth may well be part of a single process in which all the events are interdependent. Correlations and partial correlations, to measure the degree of any such interdependence, have been calculated only from animals with complete records on

all four pairs of permanent incisors, viz. 14 MZ, 14 DZ and 18 HZ (i.e. two fewer than the forty-eight animals with 4th incisors shown in Table 2—by reason of an incomplete record for the 3rd pair of teeth of two of the MZ twins).

Table 5. *Correlations and partial correlations between ages at eruption of pairs of permanent incisors*

Tooth pairs held constant	Correlation or partial correlation for pairs					
	1 : 2	1 : 3	1 : 4	2 : 3	2 : 4	3 : 4
—	0·63	0·47	0·59	0·82	0·75	0·83
1	—	—	—	0·76	0·60	0·77
2	—	−0·09	0·23	—	—	0·56
3	0·47	—	0·40	—	0·22	—
4	0·35	−0·03	—	0·54	—	—
1 and 2	—	—	—	—	—	0·60
1 and 3	—	—	—	—	0·04	—
1 and 4	—	—	—	0·59	—	—
2 and 3	—	—	0·34	—	—	—
2 and 4	—	−0·27	—	—	—	—
3 and 4	0·43	—	—	—	—	—

Had all the animals concerned been unrelated, all coefficients in Table 5 above 0·3 would have been significant at the 5% level of probability. Since the animals had various degrees of relationship, the 5% significance level will lie between 0·3 and 0·4. The corresponding values for 1% significance are 0·4 and 0·5. The outstanding features of the correlations are the comparatively high values for adjacent pairs and the lowering of the correlation between pairs when the age of eruption of intervening pairs is held constant. The negative partial correlations between pairs 1 and 3 are non-significant and are probably sampling deviations from zero.

Holding constant the age at eruption of the 1st pair of incisors appears to have little effect on the correlations between the other three pairs of teeth. Pairs 2–4 seem to form a group within which pairs 2 and 4 are equally correlated with pair 3 and not correlated with each other except through 3.

Kemball(6) has indicated that in humans if pregnancy occurs at an age prior to the eruption of the third molars the eruption of this pair appears to be accelerated. In the twin data, eruption of first and third pairs of teeth took place shortly before first and second calving respectively. There is, for the forty-six animals included in Table 5, a positive correlation of about 0·4 between age at eruption of the first pair of incisors and age at first calving and a similar correlation between the age at eruption of the third pair of incisors and age at second calving. There is no correlation between either of the two ages at calving and age at eruption of the second or fourth pairs of teeth except that expected through their direct correlation with other tooth pairs. The data therefore are in accordance with the idea that a higher metabolic rate during pregnancy(10) may speed dental development. The correlations of age at eruption with aspects of performance such as pregnancy may, in some measure, explain the relationships in Table 5.

Relationship between body weight and age at eruption

It has been claimed that beef breeds develop their incisors at an earlier age than dairy-type animals. The animals available in the present experiment were all on the same management, but were of a large variety of breeds and crosses that prevented a direct

partition of the data by breed types. However, body weight at a particular age will in part be a function of breed type as will the increase in weight over a fixed period of time. Accordingly, age at eruption of the 1st pair of incisors was correlated with weight at 18 months of age, and time between the eruption of 1st and 4th pairs of incisors with weight increase between 18 and 42 months. The results are shown in Table 6.

Table 6. *Correlation between body weight and age at eruption of incisors*

	(*a*) Weight at 18 months and age at eruption of 1st incisors		(*b*) Weight difference (42 – 18 months) and age difference (4th – 1st incisors)	
Group	No. of animals	r	No. of animals	r
MZ	30	−0·20	16	−0·22
DZ	28	−0·30	14	+0·19
HZ	26	−0·15	18	−0·26
All	84	−0·19	48	−0·10

The correlation (−0·19) between 18 months' weight and age at eruption of the 1st pair of permanent incisors does not quite reach the 5% significance level and none of the correlations in part (*b*) of Table 6 are significant. Nevertheless, the three groups in part (*a*) agree in suggesting that the heavier the animal at 18 months of age, the earlier the 1st incisors erupt. There is less evidence for a relationship between gain in weight and age difference between first and last teeth. But for the behaviour of the DZ group it could have been concluded that the more weight gained in the 2 years following 18 months of age, the shorter is the interval between eruption of first and last teeth.

If beef animals mature more quickly than dairy types and if any of the above weight relationships are a function of maturity, then the correlations are at least in the direction expected.

Interval between Eruption and complete Emergence

The data on this aspect of incisor development were analysed in the same manner as the data on age at eruption. Table 7 gives the mean intervals between eruption and complete emergence for the four pairs of incisors.

Table 7. *Interval between eruption and complete emergence*

Incisor pair	No. of animals	Mean interval (weeks)	Mean square of interval
1st	84	5·9	2·30
2nd	74	4·4	0·85
3rd	64	5·3	1·59
4th	42	9·8	7·61

The mean interval for the first three pairs of incisors between eruption and complete emergence was about 5 weeks with a standard deviation for each pair of a little over 1 week. The length of time the 4th pair took to come fully up, however, was nearly double that of the earlier teeth. The mean square for this pair (7·6) was also much greater. In some other mammals and in humans the canine teeth (i.e. the 4th pair of 'incisors' in cattle and sheep) also take longer to grow both before and after eruption than the three pairs of incisors (18). At the age of $3\frac{1}{2}$ years when in cattle this pair emerges, bone growth around the tooth has by no means ceased. Skeletal growth, however, has become very slow at this age and the behaviour of the 4th incisors accords with this fact.

Correlations between the times taken by successive pairs of teeth to emerge fully were all less than +0·5. But as before, the strongest correlations were between the second and third pairs of teeth and between the third and fourth; these were but little affected by holding constant the values for the other pairs of teeth. Correlations between pairs that were not adjacent were due almost entirely to correlations with intervening pairs of teeth.

An analysis of variance of these data showed that the smallest part of the variance was due to differences between left side and right side—although the proportion of this to the total variance was appreciably greater than for age at eruption. With the exception of the value for the 4th incisors of HZ cows, the mean squares within animals were consistent for both groups of twins and the half-sibs, eight of twelve values falling between 0·40 and 0·59. Variances between pairs were larger than those within pairs in most cases, but significantly so in less than half of them. The within-pair mean squares were greater, by and large, for DZ and HZ than for MZ pairs, but not in the striking way shown by age at eruption. If there is any effect of heredity on rate of emergence, it is small.

DISCUSSION

Variation in age at eruption of incisors reduces their value for determining age. Some animals have their second pair of teeth before others have erupted their first. One animal among the twins discussed even had a third incisor before a number of others had had their first. None of the twins in the present study had cut their 1st permanent incisors nor had their second pair fully up (or even cut) at the age suggested in the Smithfield rules. On the other hand, nine animals had cut their 3rd pair of incisors before 2 years 8 months—the stipulated age—one of them by as much as 14 weeks. Only one of the half-sibs had a 4th pair of teeth fully emerged before the age of 3 years, but the state of the anterior molars, also considered under Smithfield regulations at this age, is not known. Under the rules, an exhibitor may prove the age of his beast when its dentition is not in accordance with expectation, and this no doubt will meet most contingencies.

If this study had been made only on twin data, it would have seemed clear that such uncontrolled differences as there were between the two environments of a pair of twins reared together had a negligible effect on the age at which the several incisor teeth appeared. One-egg twins resembled each other as closely as right and left sides of all animals, whereas two-egg twins kept along with them were significantly less concordant. Both kinds of twins differed markedly from pair to pair, no doubt as a result of genetic differences between pairs. Such differences were augmented perhaps by environmental factors that assumed importance in comparisons of animals born as much as 8 months apart. In a population of animals born on the same day, reared together uniformly and characterized by a genetic variation twice that found within DZ pairs, 90% or more of the observed variation would have appeared to be genetic. Had this been the whole truth, however, the variance within HZ pairs ($e'^2+\frac{3}{4}g'^2$) should have been just $\frac{1}{4}g'^2$ larger than the variance for DZ pairs for each of the four pairs of teeth. In fact there was a discrepancy, m'^2, too large to be ignored, although in no case significant. The relative magnitude of m'^2 increased with time.

If the estimate of e'^2 and g'^2 (from twins) and m'^2 (from HZ pairs) are summed, and each expressed as a percentage of the total, as has been done in Table 8, the changes in m'^2 relative to e'^2 and g'^2 can be easily followed. For the first incisors, m'^2 was negative because

the HZ pairs were unexpectedly less variable (within pairs) than DZ pairs. Large standard errors attaching to the values of m'^2 allow this, and indeed the remaining values of m'^2, to be attributed to sampling error. The time trend which results in m'^2 amounting to 60% of the total variance for the 4th incisors is, nevertheless, suggestive of a meaning for m'^2 beyond its content of sampling errors; otherwise these errors have to be supposed to increase in an orderly fashion. This is not impossible, especially since numbers of animals were culled during the course of the observations, though for reasons unrelated to teeth. It may be recalled, however, that estimates of m'^2 from data on the growth of all animals up to 18 months of age were also rather too large to be overlooked (7). Demands on sampling errors become more heavy to sustain than those on the hypothesis that m'^2 measures a real departure from the postulate that e'^2 and g'^2 account for all variation and can be added together.

Table 8. *Estimates of e'^2, g'^2 and m'^2 expressed as percentage of their sum*

	Incisors			
	1st	2nd	3rd	4th
% e'^2	7·8	4·9	1·7	2·7
% g'^2	129·1	84·7	60·3	37·6
% m'^2	−36·9	10·4	38·0	59·7

It is difficult to suppose that m'^2 for 4th incisors has much to do with immediately pre- or post-natal effects. A more likely explanation is that it is due to a kind of genotype-environment interaction. Although superficially exposed to the same environment, all animals in this experiment had some degree of choice in respect of its components (for instance when at grass) and their own individual reactions to such of those components as were constant for all. The results are readily conceived to be cumulative so that m'^2 would increase with time. HZ pairs, being more genetically unlike, would vary most in their reactions. DZ twins would vary less, but all variation from this cause would be inseparable from additive genetic variation and would help to inflate the estimates of heritability to the high observed values. Consequently, m'^2 obtained by comparing MZ and DZ with HZ pairs is possibly an underestimate of this type of variation to the extent that e^2_{DZ} is greater than e^2_{MZ}. At present there is no direct evidence of the magnitude of e^2_{DZ} in cattle.

In human twins, e^2_{DZ} may in fact be less than e^2_{MZ}. Intra-pair variation in human monozygotic twins attributable to vascular anastomosis is claimed to be often such as to cause individual differences greater than those in dizygotic pairs where anastomosis does not occur (2). The result of ignoring this would be to underestimate the importance of heredity. This primary bias is unlikely to have a similar disturbing effect in studies of cattle twins since the great majority of dizygotic twins establish vascular anastomosis (1).

It is a relevant question whether or not the same pairs of animals contribute proportionately similar amounts to the variance of the different characters studied. The characters themselves are very likely to be dependent on each other as different pairs of incisor teeth have been shown to be, or as height and weight are. Pairs of animals, therefore, which for any reason acquire a comparatively large difference in one respect may well acquire a large difference in another.

Several considerations demand further study of this subject. The immediate problem

is how far it is justifiable to regard the evidence for the existence of m'^2 presented in this paper as confirming the earlier evidence (7) based on the growth rate of the same animals. The correlations between growth rate and age at eruption of incisor teeth given above are small and non-significant and it could therefore be held that the two bodies of evidence for m'^2 are together much stronger than either taken alone. Just how much stronger they are in combination with each other or with further evidence from the same animals depends on a more precise knowledge of the correlations between the characters studied than is yet available.

SUMMARY

1. Data are presented on variation in age at eruption of permanent incisor teeth in one-egg (MZ) and two-egg (DZ) twins and in pairs of half sisters (HZ). The 1st pair of incisors erupted when animals were on average 100 weeks old and the other three pairs came at roughly 26-week intervals thereafter. Variation in age at eruption increased from 1st to 4th incisors, but the coefficient of variation remained constant at about 8%. The mean interval between eruption and complete emergence was about 5 weeks for the first three pairs of teeth and nearly 10 weeks for the 4th pair.

2. Variation in age at eruption within MZ pairs was no greater than that between left and right sides of the same animal. DZ displayed greater within-pair variances than MZ twins, and HZ pairs greater than DZ.

3. Correlations of age at eruption of teeth with body weight and with gain in weight were negative but non-significant. Age at eruption of 1st and of 3rd teeth was positively correlated with age at 1st and 2nd calving, but eruption of 2nd and of 4th teeth was not.

4. For 2nd, 3rd and 4th incisors, the intra-pair variance of the HZ group was in excess of that expected. This can be interpreted to mean that the estimates of environmental and genetic variation derivable from MZ and DZ pairs are not adequate to account for all the variation within HZ pairs.

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REFERENCES

(1) Billingham, R. E., Lampkin, G. H., Medawar, P. B. & Williams, H. Ll. (1952). *Heredity* **6**, 201.
(2) Price, B. (1950). *Amer. J. hum. Genet.* **2**, 293.
(3) Brown, G. T. (1949). *Dentition as Indicative of the Age of the Animals of the Farm*, 9th ed. London: Murray.
(4) Donald, H. P. (1953). *J. Dairy Res.* **20**, 355.
(5) Franklin, M. C. (1950). *Bull. Coun. Sci. Industr. Res. Aust.* no. 252.
(6) Kemball, C. H. Personal communication.
(7) King, J. W. B. & Donald, H. P. (1955). *J. Dairy Res.* **22**, 1.
(8) Liddell, H. S. (1925). *Anat. Rec.* **30**, 327.
(9) Miller, W. C. & Robertson, E. D. S. (1952). *Practical Animal Husbandry*, 6th ed. Edinburgh: Oliver and Boyd.
(10) Newton, W. H. (1952). *Marshall's Physiology of Reproduction*, 3rd ed. (editor A. S. Parkes), p. 448. London: Longmans Green.
(11) Searle, A. G. (1954). *J. Genet.* **52**, 413.
(12) Simonds, J. B. (1855). *J.R. Agric. Soc.* (1st series), **15**, 276.

(13) SISSON, S. S. B. & GROSSMAN, J. D. (1953). *The Anatomy of the Domestic Animals*, 4th ed. Philadelphia: Saunders.
(14) SMITHFIELD CLUB (1954). *Prize Sheet for Smithfield Show*. Bath, Smithfield Club.
(15) STONES, H. H. (1954). *Oral and Dental Diseases*, 3rd ed. Edinburgh: Livingstone.
(16) TODD, T. W. & WHARTON, R. E. (1934). *Amer. J. Anat.* **55**, 97.
(17) TOMES, C. S. (1923). *A Manual of Dental Anatomy*, 8th ed. (editors: H. W. Marett Tims and C. Bowdler Henry). London: Churchill.
(18) WIDDOWSON, T. W. (1952). *Special or Dental Anatomy and Physiology and Dental Histology*, vol. 1, 8th ed. London: Staples Press.
(19) YOUATT, W. (1834). *Cattle, their Breeds, Management and Diseases*. London: Baldwin.
(20) ZORN, W. (1953). *Handbuch der Landwirtschaft; IV. Besondere Tierzuchtlehre*, 1. *Rindviehzucht*. 6th ed. (editor: J. Schmidt). Berlin: Paul Parey.

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580. THE DETERIORATION OF MILKING RUBBERS

IV. FAT IN MILK LINERS

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(With 2 Figures)

Following the earlier papers in this series ((1)–(4)) further work has been carried out on the fats and greases which attack milking rubbers. The sources from which they come have been investigated and an estimate made of the composition of the fatty material obtained from used milk liners.

FATTY MATERIAL FROM USED MILK LINERS

The major cause of deterioration of milk liners has long been recognized as the absorption of fats by the rubber ((2)–(18)). Robertson, Finch & Breed (5), when referring to the heat sterilization of milking rubbers, stated: 'As these rubber parts were not attached to the usual metal parts and as they were not exposed to the fats from the skin and from the milk, definite conclusions regarding the life of the rubber under field conditions ought not to be drawn from these experiments.' Hoy & Neave, in a more recent paper (12), stated: 'It seems clear that the increase in weight is due to the absorption of fat. What proportion comes from the skin of the cow and whether the fat can be removed from the rubber by washing in alkali has still to be investigated.' These authors appeared to recognize the deteriorating effects of skin and milk fats.

Some investigations have been carried out on the ethereal extract of a used milk liner by Gardner & Berridge (2). This extract contained substances which are present in a new milk liner and which would be removed by ether extraction of such a liner. In the present work, this contamination of the fatty extract was minimized, but not completely eliminated, by extraction of some milk liners before they were put into use on the farm.

Thirty-six new straight tube liners were extracted in boiling acetone for 24 hr. They were then dried in a warm atmosphere for about a fortnight before being sent to three different farms. The details of farm treatment were as follows:

Farm no. 1. Twelve liners, 3 units in use. Period of service, 7 weeks. Washed daily with detergent solution and steam sterilized once per week. Cut back once during service.

Farm no. 2. Twelve liners, 3 units in use. Period of service, 6 weeks. Washed daily in hot water and dismantled once a week for a wash in hot detergent solution. Not cut back during service.

Farm no. 3. Twelve liners, 3 units in use. Period of service, 5 weeks. Washed daily in hot detergent solution followed by steam sterilization. Dismantled once a fortnight for thorough cleaning. Not cut back during service.

The liners after return from the farms were cut into annular sections, each 1 in. in length except those from the claw tube end of the liners, which were slightly longer. The sections from the mouthpiece ends of all the liners were numbered 1. The other sections

were numbered 2 to 6 in sequence. All the mouthpiece annuli (sections 1) were extracted in boiling acetone for 24 hr. The other groups of sections were treated similarly. After distilling off the acetone, six samples of fatty material were obtained.

Using the liners from farm no. 1, measurements were made of the fat absorption of each section. The mean values for the percentage volume extracts of each section are given in Table 1. From these figures the second row of results was calculated and this shows the percentage of the total fat in each section of the liner.

Table 1

	Sections of liner						
Mouthpiece end →	1	2	3	4	5	6	← Claw tube end.
Percentage volume fatty extract	20	45	39	20	2	1	
Percentage of total fat	16	35	30	16	2	1	

As a check on the efficiency of the extraction of the new milk liners before their despatch to the farms for test, two milk liners, extracted with the original thirty-six test liners were again extracted for 24 hr. in boiling acetone. The material, which was obtained from these liners, is referred to as 'new liner extract' throughout the paper. Only a small quantity was obtained, approximately 0·6% by volume. This does demonstrate that the fatty material extracted from the liners on farm test was contaminated, but only to a small degree.

Using the extract from all the thirty-six liners on test on the three farms, the fatty material from each of the six groups of milk liner sections was tested. The density was measured by a capillary tube method. The results were corrected to 15° C. using the correction factor for butterfat, 0·0007 per ° C. (19). The refractive index was measured by the use of a sodium flame and a Leitz-Jelley refractometer to which a small heating element had been fitted. The saponification value, the saponification equivalent, the percentage unsaponifiable matter value and the melting-point value were determined by the methods laid down by the British Standards Institution (20). The melting-point result was obtained by the slip-point method.

The results of these measurements are given in Table 2. Samples of butterfat, woolfat, new liner extract and cow-udder fat were also tested, and the results of these tests are included in Table 2. Cow-udder fat was obtained from three different farms. The cows' teats were washed with acetone and the resulting mixture of solids and acetone solution was filtered while hot. The acetone was distilled off from the filtrate and the skin fat remained in the distillation flask. Altogether some 4 g. were used in the tests and this was the amount of fat from the udders of approximately 200 cows.

The results of some similar measurements for butterfat and woolfat were taken from the literature (21). These are given in Table 3.

The results given in Table 2 for butterfat and woolfat compare favourably with those given in Table 3. The density values show somewhat large differences, and therefore the column of density values in Table 2 cannot be considered to be very reliable.

In general the results in Table 2 show a trend in nearly all cases from section 1 to section 6, in which the variable reaches a maximum, a minimum or a point of inflexion at section 3 or 4. This is an important point, as it is considered that these variations are connected with the dominant effects of udder fat at section 1, butterfat at sections 3 and 4

and new liner extract at section 6. The results for refractive index, saponification value and percentage unsaponifiable matter are plotted in Fig. 1(*a*, *b*, *c*) and illustrate the observations made above.

Table 2

Fat	Appearance	Density (15°)	Refractive index (40°)	Saponification value*	Saponification equivalent*	Percentage unsaponifiable matter (ether soluble)	Precentage unsaponifiable matter (ether insoluble)	Melting-point (° C.)
Section 1	Yellow	0·911	1·463	148	379	25·2	1·5	26
Section 2	Pale yellow	0·929	1·457	182	308	16·5	Trace	27
Section 3	Pale yellow	0·932	1·457	195	288	13·8	0·2	25
Section 4	Pale yellow	0·925	1·458	200	280	9·9	1·0	26
Section 5	Yellow	0·940	1·462	166	338	20·7	1·3	32
Section 6	Deep yellow	0·960	1·471	115	488	29·5	4·5	34
New liner extract	Brown resinous material	0·965	1·488	132	425	20·2	9·3	48
Butterfat	Yellow	0·930	1·453	216	260	0·6	Trace	29
Cow-udder fat	Variable—yellow to olive green	0·913	1·462	159	353	24·4	1·1	35
Woolfat	Deep yellow	0·958	1·481	100	560	44·1	5·9	39

* See Hilditch[21], p. 40, for definitions.

Table 3

Fat	Density	Refractive index	Saponification value	Saponification equivalent	Percentage unsaponifiable matter (ether soluble)	Melting-point (° C.)
Butterfat	0·936–0·944 15°	1·460 25°	216–235	237–260	0·3–0·5	28–33
Woolfat	0·941–0·945 15·5°	1·480 40°	100–110	(510–560)*	35–50 (alcohols)	31–42

* The brackets indicate that this value was calculated ignoring the large percentage of unsaponifiable matter.

A rough calculation of the percentage of cow-udder fat and the percentage of butterfat in each section of the used liners has been made. The results given in Table 1 were used for the distribution of fat in the liners. The results for refractive index, saponification value and percentage unsaponifiable matter were used, and the calculation was made on the assumption that the proportion of cow-udder fat to butterfat could be obtained from a linear graph. (The effect of the small percentage of 'new liner extract' was ignored.)

Fig. 2 is an example and shows the graph drawn for saponification value against percentage butterfat and cow-udder fat. In Table 4, the percentage values for the two types of fat in each section are given.

Table 4

	Refractive index		Saponification value		Unsaponifiable matter		Mean values	
	Percentage		Percentage		Percentage		Percentage	
Sections	Cow-udder fat	Butterfat	Cow-udder fat	Butterfat	Cow-udder fat	Butterfat	Cow-udder fat	Butterfat
1	16	0	16	0	16	0	16	0
2	16	19	20	15	23	12	20	15
3	13	17	12	18	16	14	14	16
4	9	7	5	11	6	10	7	9
5	0	2	0	2	0	2	0	2
6	0	1	0	1	0	1	0	1
	54	46	53	47	61	39	57	43

These calculations indicate that as much as 50–60% of the fatty material absorbed by a milk liner may be from the skin and hair of the cow at the junction of the teats and udder. The whole of the fat present in the mouthpiece of a liner appears to be from this source. In the barrel where the teat is enclosed during milking, both fat from the milk and from the cow's skin and hair is absorbed by the liner.

These results indicate that a thorough washing of the udder with a warm detergent

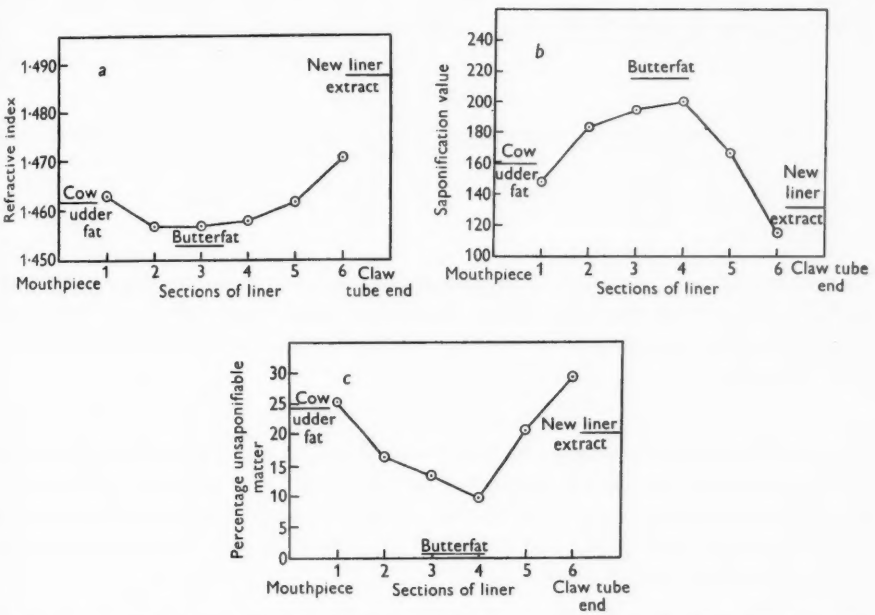

Fig. 1 (*a*). Refractive indices of fats from different sections of liner. 1 (*b*). Saponification values of fats from different sections of liner. 1 (*c*). Percentage unsaponifiable matter of fats from different sections of liner.

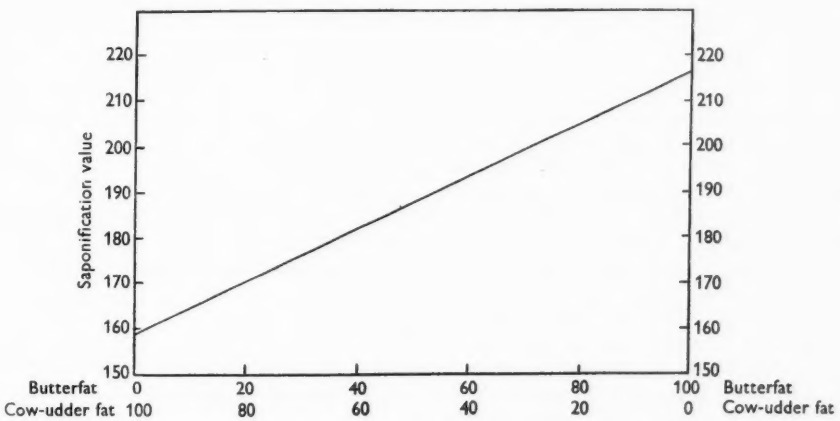

Fig. 2. Assumed graph for saponification values of mixtures of butterfat and cow-udder fat.

solution at fairly frequent intervals should reduce the rate of fat absorption. The use of paper towels as recommended by Watson (22) is considered to be sound. If udder salves are used, they should not be applied just before milking (3, 23).

COW-UDDER FAT

As investigations on cow-udder fat do not appear to have been made previously, it was decided to give the individual results obtained from the three samples of fat used. These figures are given in Table 5. The mean values were given in Table 2.

Table 5

Density (15°)	Refractive index (40°)	Saponification value	Saponification equivalent	Percentage unsaponifiable matter (ether soluble)	Melting-point (° C.)
0·906	1·463	159	353	23·6	22
0·913	1·461	174	322	25·0	39
0·921	1·462	143	392	24·7	44

The refractive index and percentage unsaponifiable matter values gave reasonably close results for the three samples. The melting-point values were widely different. One sample was an olive green greasy material, while the other two were solid, yellowish green fats at room temperature.

CONCLUSIONS AND SUMMARY

The analysis of the fatty extracts from used milk liners has shown that the fat absorbed in the mouthpiece of a liner is mainly fat from the cows' skin and hair, while the fat absorbed in the barrel is a mixture of butterfat and cow-udder fat. The percentage of each type of fat varies but it would appear that 50–60% of the fat absorbed by a liner on the farm may be from the cow's skin and hair.

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REFERENCES

(1) Berridge, N. J. (1951). *J. Dairy Res.* **18**, 246.
(2) Gardner, E. R. & Berridge, N. J. (1952). *J. Dairy Res.* **19**, 31.
(3) Cooper, J. H. & Gardner, E. R. (1953). *J. Dairy Res.* **20**, 340.
(4) Clarke, P. M., Gardner, E. R. & Berridge, N. J. (1954). In the Press.
(5) Robertson, A. H., Finch, M. W. & Breed, R. S. (1922). *Bull. N.Z. Agric. Exp. Sta.* no. 492.
(6) Burgwald, L. H. (1923). *U.S. Dep. Agric. Farmers' Bull.* no. 1315.
(7) Lochhead, A. G. & Johns, C. K. (1929). *Bull. Dom. Can. Dep. Agric.* no. 127.
(8) Parfitt, E. H. (1931). *Purdue Univ. Bull.* no. 348.
(9) Johns, C. K. (1933). *Sci. Agric.* **7**, 460.
(10) Smith, J. W. (1934). *N.Z. J. Agric.* **49**, 71.
(11) Sheldon, W. H. & Jensen, J. M. (1941). *Quart. Bull. Mich. Sta. Coll.* no. 23.
(12) Hoy, W. A. & Neave, F. K. (1941). *Proc. Soc. Agric. Bact.* (*Abstr.*), no. 39.
(13) Moir, G. M. (1943). *N.Z. J. Agric.* **67**, 153.
(14) Whittleston, W. G. (1945). *N.Z. J. Sci. Tech.* (A), **26**, 223.

(15) WHITTLESTON, W. G. (1945). *N.Z. J. Sci. Tech.* (A), **26**, 382.
(16) JENSEN, J. M. & BORTREE, A. L. (1946). *J. Dairy Sci.* **29**, 849.
(17) JOHNS, C. K. (1951). *J. Milk Fd. Tech.* **14**, 153.
(18) WHITTLESTON, W. G. (1952). *J. Soc. Dairy Tech.* **5**, 177.
(19) DAVIS, J. G. & MACDONALD, F. G. (1953). *Richmond's Dairy Chemistry*, 5th ed. p. 488. London: Charles Griffin and Co. Ltd.
(20) *Methods of Analysis of Oils and Fats*, British Standards Institution, B.S. 684 (1950).
(21) HILDITCH, T. P. (1949). *The Industrial Chemistry of Fats and Waxes*, 3rd ed. pp. 145, 148. London: Baillière, Tindall and Cox.
(22) WATSON, E. M. (1954). *Pwr. Fmr. Publ.* **12**, 34.
(23) LOW, S. W. (1951). *N.Z. J. Agric.* **82**, 229.

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581. THE DETERIORATION OF MILKING RUBBERS

V. THE EFFECT OF RUBBER COMPOSITION

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The importance of fat absorption as a cause of deterioration in the rubber teat-cup liners of milking machines has already been demonstrated (1, 2), and it is well known that the composition of the rubber affects its resistance to fat. It was considered desirable, therefore, to see what changes in composition could usefully be made, and how these would affect fat absorption under normal milking conditions. To this end, and with the co-operation of a large number of farms and Milk Production Officers, the four experiments reported here were carried out.

INTRODUCTION

In a vulcanized rubber there are a number of constituents apart from the rubber hydrocarbon. Sulphur is present for vulcanization, and among the other auxiliary materials employed, accelerators, antioxidants, oils and fillers are the most important. Accelerators, which are usually organic materials, are needed to give rapid vulcanization and so to make efficient use of processing equipment. Antioxidants, as their name suggests, improve the resistance of the rubber to deterioration by oxidation. Both oils and fillers may be used to facilitate manufacturing processes, and both types of material alter the physical properties of the vulcanized rubber. Fillers, which usually consist of finely divided inorganic materials or finely divided carbon, are added to most rubber mixes, since a vulcanized rubber containing all rubber hydrocarbon, except for the vulcanizing agents, is too soft and flexible for most purposes. The amount of stiffening achieved depends upon the type and quality of filler used.

In the first two farm tests (A and B) described below, a number of different natural rubber compositions were tested. The types of accelerator and filler, and also the quantity of inorganic filler used, were varied, subject to the limitations of having a suitable stiffness for the proper functioning of the milking machine and of ensuring a reasonably high fat resistance. Even with these restrictions, the rubber compounds tested could represent only a few of the many possible variations.

A number of synthetic rubbers are also available. These may be divided into two groups. The members of the first group, called general purpose synthetic rubbers, are used merely as replacements for natural rubber and are employed only when natural rubber is not available or for economic or strategic reasons. In some respects these rubbers are inferior to natural rubber, and some war-time teat-cup liners, which contained a proportion of this type of rubber, gave relatively poor service life. The main causes of failure were cracking due to fatigue and splitting during assembly or heat sterilization. The other

group of synthetic rubbers, called special-purpose synthetic rubbers, are employed because they show some advantage over natural rubber under certain conditions of use. Some of the rubbers of this type have a considerably greater resistance to swelling by fats and oils than natural rubber. It is evident, therefore, that liners made from these rubbers, unless they exhibit undesirable properties not shown by natural rubber, would give longer service life than those made from natural rubber.

Neoprene and Perbunan are both oil-resistant rubbers of the second type, but Neoprene has the disadvantage of a greater tendency to permanent set than natural rubber, so that liners made of this material become misshapen quite quickly, the orifice becoming enlarged and the barrel tending to assume the shape of the teat. As a result, after a short period of use, the vacuum is held only with difficulty, and trouble is experienced with cups slipping from the teats. Perbunan does not suffer from this defect to nearly the same extent, and it is possible to make liners from this rubber that outlast natural rubber liners. We are here using 'Perbunan' as a generic term to include a number of brands of butadiene-acrylonitrile rubber with a high content of butadiene. During the early part of the life of the liners the surfaces are easier to clean than are those of liners at present in general use because there is much less swelling. However, if the length of service is greatly prolonged, cleaning difficulties are encountered due to swelling and also to accumulation of milkstone. It is more difficult to manufacture Perbunan liners than the present natural rubber ones, and to assist the processes of manufacture a proportion of natural rubber is often mixed with Perbunan. Even so, the cost of manufacture is higher than for natural rubber liners, and this, with the extra cost of raw materials, makes Perbunan compound liners more expensive.

Perbunan liner compounds are not as strong as similar natural rubber compounds, and the difference increases with increase in temperature. Liners of the type which are stretched over a metal ring before assembly in the cup, if made from a compound containing a high proportion of Perbunan, tend to split in the highly stretched area when subjected to steam sterilization. This difficulty can be overcome by increasing the natural rubber content, although this, of course, also diminishes the fat resistance.

PRELIMINARY LABORATORY EXPERIMENTS

A range of mixes of natural rubber and Perbunan, with the rubber portion varying from 100% natural rubber to 100% Perbunan in steps of 10%, was examined in the laboratory. The natural rubber compound was identical with one in general use for the manufacture of liners. The auxiliary materials in the Perbunan-containing mixes were as nearly as possible the same, the main differences being in the sulphur and accelerator contents. It is usual to add less sulphur and more accelerator to a Perbunan mix than is customary with a natural rubber mix, so in this range of compounds the sulphur was decreased and the accelerator increased regularly in passing from the natural rubber to the Perbunan mix.

Column 2 in Table 1 shows the Mooney plasticity of the unvulcanized compounds. This measurement reflects the processibility of the unvulcanized material, a higher figure indicating a tougher, less easily worked stock. The increased difficulty in processing as the Perbunan content was increased is well illustrated. Table 1 also shows the progressive increase in resistance to oil and to fat after vulcanization obtained by progressively replacing natural rubber in the mixture by Perbunan.

Following these laboratory tests a number of compounds with a ratio of natural rubber to Perbunan varying from 60/40 to 0/100 by weight was developed, and the liners made from these compounds were used in farm tests C and D.

Table 1. *The effect of Perbunan on plasticity and oil resistance of rubber mixes*

Perbunan content (percentage total hydrocarbon)	Mooney plasticity (before cure)	Percentage volume increase in mineral oil*		Percentage weight increase in butterfat†	
		Cure 15 min. at 289° F.	Cure 25 min. at 298° F.	Cure 15 min. at 298° F.	Cure 25 min. at 298° F.
0	14	108	101	96	91
10	23	106	105	97	90
20	23	94·5	88·5	79	79
30	21	80	83	72	72
40	21	66·5	57·5	64	57
50	22	43·5	56	48	46
60	26	42	44	37	37
70	27	29·5	29·5	25	25
80	35	16·5	16·5	15	16
90	45	6·5	6	7	3
100	60	1·5	1·5	3	2

* Samples immersed in mineral oil for 7 days at 70° C.

† Samples immersed in butterfat for 9 days at 37° C., being removed and steamed at atmospheric pressure for 15–30 min. on alternate days.

EXPERIMENTS ON FARMS

Types of rubber used

The experiments to be described fall into two groups: Exps. A and B were carried out with natural rubber mixes only, with variations in the accelerators and fillers, while Exps. C and D were mainly concerned with mixes of natural rubber and Perbunan. Straight-tube liners were tested in Exps. A and C, and moulded liners in Exps. B and D. Table 2 summarizes the types of rubber used, showing the main variations from the normal recipe.

Table 2. *Summary of rubber compositions used in farm trials*

Exps. A (straight-tube) and B (moulded)

Composition no.	Composition
1	Normal
2	Diphenylguanidine accelerator
3	Dibenzthiazyl disulphide accelerator
4	Carbon black loading
5	Activated chalk loading, 90% rubber content by volume
6	Activated chalk loading, 85% rubber content by volume
7*	Activated chalk loading, 80% rubber content by volume

* Exp. A only.

Exps. C (straight-tube) and D (moulded)

Composition no.	Composition	
1	Normal	Normal* (A)
2	Normal, stored for 6 months before use	Normal (B)
3	100%† Perbunan, black loading	Normal, surface treated
4	80% Perbunan, black loading	80% Perbunan, clay loading
5	60% Perbunan, black loading	60% Perbunan, black loading
6	50% Perbunan, clay loading	50% Perbunan, clay loading
7	40% Perbunan, black loading	40% Perbunan, black loading

* The normal (A) rubber was from the same bulk as the normal, surface-treated rubber; the other five compositions were from another bulk of raw rubber. In each of the other experiments a single bulk of raw rubber was used.

† These values denote the percentages of Perbunan in the mixture of natural rubber and Perbunan alone, exclusive of other materials.

Plan and design

The farms participating in these experiments were drawn from many areas of the country, and were considered to be representative of average farms in respect of size and management; care was taken to avoid the natural bias towards exceptionally clean and carefully run establishments. The choice was, however, limited by the experimental design to farms with at least three milking units. It was desired to test different types of rubber on each farm, so that comparisons between rubber mixtures could be made within farms, and to do this a balanced incomplete block design, proposed and drawn up by Mr M. J. R. Healy of Rothamsted Experimental Station, was used for each of the four experiments.

Table 3. *Treatment groups and replications*

Experiment	A	B	C	D
Number of compositions (i.e. treatments)	7	6	7	7
Number of treatment groups	7	10	7	7
Intended number of replications				
(i) Of each treatment group	9	6	10	10
(ii) Of each treatment	27	30	30	30

Blocks were defined by farms, and there were three rubber compositions (i.e. treatments) in each block, so that each farm received twelve liners, four of each of three compositions. Within one experiment, each group of three treatments was equally replicated, and these groups were allocated randomly to farms. Farms receiving the same three types of liner will be referred to as a farm group. Each farmer was supposed to use equally each of the three types of liner sent him, and he was particularly requested to continue his normal routine for milking and management. The numbers of treatment groups and the intended number of replications in the four experiments are shown in Table 3.

Observations

Interest centred chiefly on fat absorption as a criterion of fitness for use, and an estimate of the fat absorbed was made for each set of liners. Additionally, estimates were made of the increases in length and diameter of the liners to give approximate comparisons of distortion. The resistance to ozone cracking was judged by visual inspection. Resistance to flexing in the short milk tube of a moulded liner is clearly important, and it is in this

Table 4. *Plan of observations*

Experiment	A	B	C	D
Observations				
(*a*) Fat content	*	*	*	*
(*b*) Increase in length (mm.)	*	—	—	—
(*c*) Increase in average diameter (mm.)	*	—	—	—
(*d*) Number of flexings before perforation	—	*	—	*
(*e*) Degree of ozone cracking	—	*	—	*

part of the liner that perforations sometimes occur. The number of flexings which used liners of the moulded type could endure before perforation occurred was therefore determined. Any remarks made by the farmers on the efficiency of the liners were recorded.

The observations made in each experiment are shown by asterisks in Table 4.

Number of samples

The linear measurements (*b*) and (*c*) (Table 4) were made on all four liners of each set, before fat extraction or flexing. In Exps. B and D, two of the four liners of a set, selected at random, were tested for fat content and examined for ozone cracking, while the other two liners were tested for resistance to flexing. In Exps. A and C all four liners of each set were tested for fat content.

Methods of measurement

The fat content of the discarded liners was measured by extracting them in batches with acetone in a large Soxhlet type of apparatus. They were weighed before and after extraction and drying. Since the new rubber contains material extractable by acetone the figures were corrected by subtracting values estimated from determinations on similar compositions. To facilitate the extraction of the moulded liners, the hoods and short milk tubes were first cut off and discarded.

In order to estimate the increases in length, the liners were marked with hot wires at a fixed distance apart (46 mm.) before being sent out, and when they were returned the distance between the marks was measured. The maximum and minimum diameters after use were measured at the point of greatest tension, i.e. at the position of the mouthpiece ring, and the average of these two measurements was taken.

The flexing apparatus was made as simple as possible. The first part was an electrically driven machine capable of bending the short milk tubes through an angle of about 130°, at the same time maintaining sufficient longitudinal tension to ensure a sharp bend and collapse of the tube at the angle, as happens in milking practice when the clusters are allowed to hang from the claw to cut off the vacuum. Eight liners were mounted in a line on this machine, using solid metal rods the size and shape of the tubes of the claw piece of a milking machine. The second part of the apparatus consisted of a vacuum pump, manostat and manometer with electrical connexions. This was connected to the interiors of the liners through their wide ends by means of rubber bungs, tubing and a manifold. The third part of the apparatus was a 7-day, 24-hr. electric clock with alarm. The whole apparatus was supplied with electric power via a relay which was controlled by the electrical contacts of the manometer. When air leaked into the system through a perforated liner, the flexing device and the clock stopped and the alarm sounded. The operator then recorded the clock reading and the designations of the liner removed and of the new one substituted. Since the flexing machine worked at a constant rate (86 flexings per min.) the number of flexings endured by each liner could be calculated.

The short milk tubes which had been cut from the moulded liners before fat extraction were examined for ozone cracking. (For a discussion of ozone cracking see, for example, Cooper & Gardner (3).) Marks were awarded on a 0 to 5 scale, between no cracking at 0 and very bad cracking at 5.

Deviations from plan

In most cases the numbers of cows being milked during the experiment changed frequently, and estimates had to be obtained by occasional visits of the Milk Production Officers. Only approximate figures could be calculated and close control was impossible. Because of a few cases of incomplete returns and unequal use of different sets on the same farm, equality in the number of replications within an experiment was not achieved, and

this led to some complication of the analysis, as described later. The numbers of farms for which full results were available, and the ranges and means of the number of milkings per liner are given in Table 5.

Table 5. *Numbers of farms completing the experiment in each group*

Experiment ...	A	B	C	D
Treatment group	Number of farms			
1	5	4	7	5
2	7	2	7	6
3	5	3	3	3
4	7	5	3	4
5	6	3	8	4
6	6	5	4	3
7	5	5	4	7
8	—	4	—	—
9	—	5	—	—
10	—	4	—	—
Total	41	40	36	32
Number of milkings per liner (hundreds):				
Range	2–19	6–26	4–18	10–52
Mean	11	20	10	24

Methods of statistical analysis

The statistical analysis was complicated by the unequal replication of the different treatment groups within an experiment. Least squares analyses, fitting constants, were carried out, using an angular transformation for the ozone cracking marks and percentages of liners perforated.

In some cases, following common practice, straight-tube liners which had stretched during use were shortened by the farmer. Accordingly, for the comparison of results in Exps. A and C, the weight of fat extracted from each liner was expressed as a percentage of the final weight of the fat-free liner, but for Exps. B and D (moulded liners) the figures for total weight of extracted fat per liner were analysed.

For Exp. D on moulded liners, the percentages of liners perforated before 30*, 1000, 2000 and 3000 flexings were calculated for each rubber mixture over the farms in each group, and analyses of variance of these percentages were carried out. The corresponding results for Exp. B, however, were not considered sufficiently reliable to justify analysis of any more than the percentages of liners holed before thirty flexings.

Differences between numbers of milkings, rest periods and methods and frequencies of sterilization were confounded with differences between farms. The variance between farms within farm groups was split up in each analysis to examine the components due to linear regression on the number of milkings and to other differences in usage and differences in sterilization methods. For Exp. A, partial correlation coefficients between pairs of the variables, fat percentage, increase in length, increase in average diameter and number of milkings, were calculated for each rubber composition.

* A value arbitrarily chosen as indicating probable but not obvious perforation when received.

RESULTS

Tables 6 and 8–10 in the following sections give the mean values (for each liner composition) of the fat content, increase in length, increase in average diameter, marks for degree of ozone cracking and percentages of liners holed before 30, 1000, 2000 and 3000 flexings.

Table 6. *Mean values for Exp. A*

Composition	No. of farms	Fat percentage		Increase in length (mm.)		Increase in average diameter (mm.)	
		Mean	Difference from normal rubber	Mean	Difference from normal rubber	Mean	Difference from normal rubber
1. Normal rubber	17	13·07	—	4·62	—	3·32	—
2. Diphenylguanidine accelerator	18	15·89	2·82***	4·79	0·17	3·39	0·07
3. Dibenzthiazyl disulphide accelerator	20	14·88	1·81**	5·62	1·00**	3·44	0·12
4. Black loading	16	12·41	−0·66	3·63	−0·99**	2·77	−0·55**
5. Activated chalk loading, 90% rubber	17	13·94	0·87	4·62	0·00	2·27	−1·05***
6. Activated chalk loading, 85% rubber	17	11·34	−1·73**	2·90	−1·72***	2·65	−0·67***
7. Activated chalk loading, 80% rubber	18	10·64	−2·43***	3·47	−1·15***	2·50	−0·82***
Mean	—	13·17	—	4·24	—	2·91	—
Standard error (76 D.F.)	—	—	0·62	—	0·33	—	0·18

** $P<0·01$; *** $P<0·001$.

Table 7. *Coefficients of partial correlation between pairs of the variables fat percentage, increase in length and increase in average diameter, holding the third variable and the number of milkings constant (Exp. A)*

Composition	Degrees of freedom	Fat percentage and increase in length	Variables, fat percentage and increase in diameter	Increases in length and diameter
1. Normal rubber	13	0·665**	0·303	0·350
2. Diphenylguanidine accelerator	14	0·656**	0·104	0·563*
3. Benzthiazyl disulphide accelerator	16	0·772***	0·399	0·783***
4. Black loading	12	0·775**	0·171	0·339
5. Activated chalk loading, 90% rubber	13	0·848***	0·370	0·550*
6. Activated chalk loading, 85% rubber	13	0·756**	0·009	0·413
7. Activated chalk loading, 80% rubber	14	0·510*	0·326	0·241

* $P<0·05$; ** $P<0·01$; *** $P<0·001$.

These means have been adjusted to allow for differences between farms. The mean values for ozone cracking and percentages holed were derived from the means of the transformed values. For each rubber composition, the number of replications is also shown. Because of the unequal replication and the non-orthogonality, means for different compositions have slightly different standard errors, and approximate average standard errors have been presented in the tables for use with treatment comparisons. The differences between the largest and smallest standard errors for any one set of comparisons are so small that use of these average values does not appreciably affect results of tests of significance.

Coefficients of partial correlation between percentage fat absorbed and increases in length and diameter are given in Table 7.

The mean squares in the analyses of variance are shown in the Appendix, Tables 1*a*–4*a*. Owing perhaps to the small numbers of comparable results, no significant differences between the means for different rest periods and methods of sterilization, adjusted for number of milkings, were found, and differences between rest periods and methods of sterilization were ignored in the final analyses of results.

Experiment A

As seen from Table 6, each of the two accelerators on test gave an increase in the percentage of fat absorbed, compared with the performance of the normal rubber, and the increase in length of the rubber containing dibenzthiazyl disulphide was greater than that of the normal rubber. The difference in percentage fat absorption between the two compositions containing these accelerators was not significant ($P \simeq 0{\cdot}10$).

Table 8. *Mean values for Exp. B*

Composition	No. of farms	Fat content (g./liner) Mean	Fat content (g./liner) Difference from normal	Ozone cracking (marks out of 5)† Mean	Ozone cracking (marks out of 5)† Difference from normal	Percentage holed† Mean	Percentage holed† Difference from normal
1. Normal rubber	23	3·70	—	3·04	—	33·2	—
2. Diphenylguanidine accelerator	21	5·04	1·34***	2·06	−0·98***	40·1	6·9
3. Dibenzthiazyl disulphide accelerator	18	4·10	0·40	3·50	0·46	27·0	−6·2
4. Black loading	18	2·97	−0·73*	3·38	0·34	31·6	−1·6
5. Activated chalk loading, 90% rubber	21	3·46	−0·24	2·96	−0·08	39·6	6·4
6. Activated chalk loading, 85% rubber	19	2·24	−1·46***	3·22	0·18	41·3	8·1
Mean	—	3·58	—	3·03	—	35·4	—
Standard error (75 D.F.)	—	—	0·31	—	‡	—	‡

* $P < 0{\cdot}05$; *** $P < 0{\cdot}001$.
† Values derived back from means of transformed values.
‡ Results of tests of significance apply to differences between means of transformed values.

Table 9. *Mean values for Exp. C*

Composition	No. of farms	Fat percentage Mean	Fat percentage Difference from normal rubber
1. Normal rubber	17	16·64	—
2. Normal rubber, stored for 6 months	15	14·42	−2·22*
3. 100% Perbunan, carbon black loading	10	4·66	−11·98***
4. 80% Perbunan, carbon black loading	15	2·48	−14·16***
5. 60% Perbunan, carbon black loading	14	4·98	−11·66***
6. 50% Perbunan, clay loading	19	13·03	−3·61***
7. 40% Perbunan, carbon black loading	18	12·21	−4·43***
Mean	—	9·77	—
Standard error (66 D.F.)	—	—	1·05

* $P < 0{\cdot}05$; *** $P < 0{\cdot}001$.

The rubbers containing activated chalk or black loading, on the other hand, in general increased less in length and diameter than the normal rubber, and as the percentage of chalk loading was increased, the percentage fat absorption decreased to a value 2·4% below that for normal rubber.

Examination of the variation between farms using the same combinations of rubber compositions showed that the percentage of fat extracted from the liners increased with the number of milkings for which the liners had been used, confirming the observations of Cooper & Gardner (3). The increases in length and diameter of the liners also increased with the number of milkings, although for the former measurement the linear regression coefficient was not significant. The regression coefficients for all three variables, expressed per hundred milkings, were as follows:

Fat percentage, $0{\cdot}716 \pm 0{\cdot}348$ ($P < 0{\cdot}05$).
Increase in length (mm. per liner), $0{\cdot}180 \pm 0{\cdot}109$ ($P > 0{\cdot}10$).
Increase in diameter (mm. per liner), $0{\cdot}169 \pm 0{\cdot}068$ ($P < 0{\cdot}02$).

Partial correlation coefficients were calculated for each rubber composition separately to investigate the relation between the fat absorbed and the stretching of the liners. Table 7 shows, for each composition, the coefficients which measure the correlation between pairs of the variables fat percentage, increase in length and increase in average diameter when the third variable and the number of milkings are held constant.

Table 10. *Mean values for Exp. D*

Composition	No. of farms	Fat content (g./liner) (mean)	Ozone cracking (marks)† (mean)	Percentage of liners perforated before 30 flexings† (mean)	1000 flexings† (mean)	2000 flexings† (mean)	3000 flexings† (mean)
1. Normal rubber (A)‡	14	4·23	2·39	11·7	65·6	97·8	100·0
2. Normal rubber (B)	17	4·92	1·93	15·5	76·6	100·0	100·0
3. Normal rubber, surface treated	14	4·31	0·68	8·7	52·4	90·3	97·1
4. 80% Perbunan, clay loading	13	2·30	0·42	24·8	51·9	97·7	100·0
5. 60% Perbunan, carbon black loading	10	0·66	0·77	11·1	40·5	68·1	78·5
6. 50% Perbunan, clay loading	13	2·88	0·51	15·6	49·8	69·9	94·0
7. 40% Perbunan, carbon black loading	15	2·67	1·80	14·5	35·0	74·6	97·9
Mean	—	3·14	1·13	14·2	53·4	72·6	95·4

Composition	Difference from normal rubber: Fat content (g./liner) (mean)	Ozone cracking (marks)† (mean)	Percentage of liners perforated before: 30 flexings† (mean)	1000 flexings† (mean)	2000 flexings† (mean)	3000 flexings† (mean)
3. Normal rubber, surface-treated	+0·08	−1·71**	−3·0	−13·2	−7·5	−2·9
4. 80% Perbunan, clay loading	−2·62***	−1·51**	+9·3	−24·7	−2·3	+0·0
5. 60% Perbunan, carbon black loading	−4·26***	−1·16	−4·4	−36·1*	−31·9	−21·5
6. 50% Perbunan, clay loading	−2·04***	−1·42*	+0·1	−26·8*	−30·1	−6·0
7. 40% Perbunan, carbon black loading	−2·25***	−0·13	−1·0	−41·6**	−25·4	−2·1
Standard error (58 D.F.)	0·39	§	§	§	§	§

* $P<0·05$; ** $P<0·01$; *** $P<0·001$.

† Values derived back from means of transformed values.

‡ The normal (A) rubber was from the same bulk as the normal surface-treated rubber; the other five compositions were from another bulk of rubber.

§ Results of tests of significance apply to differences between means of transformed values.

For all seven compositions the partial correlation between percentage fat extracted and increase in length was significant ($P<0·05$), but that between percentage fat and increase in diameter was in no case significant at the 5% level. There was, for three of the seven compositions, a significant partial correlation between the increase in length and the increase in diameter ($P<0·05$).

The partial correlation coefficients between pairs of variables for different compositions, presented in Table 7, are not easily comparable since they are not independent. Comparisons, however, between partial correlation coefficients for different compositions calculated only from independent observations, i.e. from results from different farms, gave no evidence of significant differences between corresponding correlation coefficients for different rubbers.

Experiment B

The results in this experiment were in general similar to those of Exp. A, as may be seen from Table 8. Noteworthy points are the lessening of ozone cracking in the presence of diphenylguanidine accelerator and the generally high percentage of liners returned with perforations.

Differences of fat absorption between farms in the same farm groups were highly significant ($P < 0{\cdot}001$) even after adjustment of the farm means to allow for differences between numbers of milkings. The coefficient of linear regression of fat content on number of milkings was $0{\cdot}225 \pm 0{\cdot}091$ g. per liner per hundred milkings ($P < 0{\cdot}05$).

The coefficient of linear regression of ozone cracking value on number of milkings was not significant ($P > 0{\cdot}05$); differences between farms in the same farm groups were, however, highly significant ($P < 0{\cdot}001$).

Experiment C

The outstanding feature of the results of this experiment, which are summarized in Table 9, was the relatively low percentage of fat absorbed by the three rubbers with the highest percentage of Perbunan. A lower mean percentage of fat was extracted from each of the test rubbers than from the normal rubber. Apart from a drop in mean fat percentage between the 100% Perbunan and 80% Perbunan ($P < 0{\cdot}05$), the mean value for the black-loaded rubbers increased for decreased percentage of Perbunan. The mean value for the 50% Perbunan with clay loading was higher than that for the 40% Perbunan with black loading, but this difference was not significant at the 5% level.

Some of the 100% Perbunan liners broke during sterilization by heat, so that some farmers who employed this method of sterilization returned this particular set of liners unused, or little used. For comparative purposes, results for farms where the different types of liner had received unequal use were excluded from the analysis, and the mean values in Table 9 should be comparable from the point of view of numbers of milkings.

Differences between farms, when treatment differences had been eliminated, were highly significant ($P < 0{\cdot}001$); although there was an increase in percentage fat for increased number of milkings, the linear regression coefficient was not significant ($b = 0{\cdot}305 \pm 0{\cdot}204$% per hundred milkings, $P > 0{\cdot}05$).

Experiment D

In this experiment also, all four Perbunan mixtures showed a greater resistance to fat than the normal rubber. The rubber with 60% Perbunan and carbon black was outstanding in its resistance to fat, and was also better than or as good as normal rubber in the other properties examined. The Perbunan mixtures with clay loading showed less ozone cracking than the normal rubber. Surface treatment of normal rubber also appeared to reduce ozone cracking but had no apparent effect on fat absorption.

When differences between treatments had been eliminated, differences of fat absorption between farms were found to be highly significant ($P < 0{\cdot}001$). The coefficient of linear regression on the number of milkings was again positive, but only reached the 6% level of significance ($b = 0{\cdot}061 \pm 0{\cdot}031$ g. per liner per hundred milkings).

Differences between the mean scores for ozone cracking on farms using the same rubber compositions were highly significant ($P < 0{\cdot}001$); there was no significant regression on the number of milkings ($P > 0{\cdot}05$).

CONCLUSIONS

In these tests, carried out under operational conditions, of rubber liners made of different compositions, the great improvement in resistance to fat obtained by replacing some or all of the natural rubber by Perbunan contrasts strongly with the trifling advantages in

this respect gained by small modifications in the formulation of natural rubber mixes. Some increase in the working life of the liners would almost certainly result from such reductions in fat absorption, although these experiments do not show any clear differences in resistance to flexing. It is therefore considered important to discover if there is in general an increase in operational life when Perbunan is used, and whether it is sufficient to justify the greater expense of manufacture. This could be done in an experiment designed primarily to investigate length of service rather than fat absorption.

Of the natural rubber mixes, those containing either of the two non-standard accelerators tested absorbed more fat than did the normal rubber, whereas carbon black or activated chalk loading in general gave reductions in fat absorbed and in the permanent set of the liners.

There was an indication that carbon black may be a more satisfactory loading material than clay for mixes incorporating Perbunan.

From these experiments, information was obtained on other points besides the relative performances of the different compositions. Among these is additional confirmation (see also (1)) of the belief that the amount of fat absorbed by liners increases with the number of milkings, more clearly demonstrated in the experiments with natural rubbers than in the experiments with Perbunan. In the experiment on straight-tube liners made of natural rubber, the examination of the linear measurements made showed a strong correlation between the percentage, by weight, of fat extracted after use and the increase in length. The corresponding correlation between percentage fat and increase in diameter was not significant ($P > 0{\cdot}05$), although there was some evidence of a positive correlation between increases in length and diameter.

There was more variation between farms than within farms in the estimates of fat absorbed, even after allowing for differences in the numbers of milkings for which the liners had been used. This variation was no doubt largely due to different methods of sterilization of the equipment and to differences in standards of cleanliness. The experiments had not been planned to investigate the effects of methods of sterilization, and the many methods represented on the participating farms were unevenly distributed with respect to rubber compositions, so that a sensitive test of this point was not available. The further experimentation suggested above could usefully be designed to incorporate a comparison of different sterilization and cleansing practices.

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REFERENCES

(1) Moir, G. M. (1943). *N.Z. Dairy Prod. Exptr*, **19**, 29.
(2) Gardner, E. R. & Berridge, N. J. (1952). *J. Dairy Res.* **19**, (1), 31.
(3) Cooper, J. H. & Gardner, E. R. (1953). *J. Dairy Res.* **20**, (3), 340.

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APPENDIX TABLES

Mean squares in the analyses of variance

Table 1*a*. *Experiment A*

Source of variation	Degrees of freedom	Mean squares: Fat percentage	Increase in length	Increase in diameter
Farm groups (ignoring treatments)	6	39·422	9·808	7·355
Farms within groups	34	164·573***	15·428***	6·617***
Linear regression on number of milkings	1	637·493*	40·118	35·667*
Residual	33	150·242***	14·680***	5·737***
Treatments (eliminating farms)	6	48·284***	12·256***	3·195***
Error	76	2·716	0·778	0·237

* $P<0·05$; *** $P<0·001$.

Table 2*a*. *Experiment B*

Source of variation	Fat content D.F.	Fat content Mean square	Ozone cracking marks D.F.	Ozone cracking marks Mean square	Percentage holed D.F.	Percentage holed Mean square
Farm groups (ignoring treatments)	9	14·892	9	1408·83	9	619·39
Farms within groups	30	17·037***	30	735·04***		
Linear regression on number of milkings	1	89·346*	1	919·90		
Residual	29	14·544***	29	728·67***		
Treatments (eliminating farms)	5	14·386***	5	594·19***	5	48·22
Error	75	0·836	75	70·72	15	207·78

* $P<0·05$; *** $P<0·001$.

Table 3*a*. *Experiment C*

Source of variation	Degrees of freedom	Mean square (fat percentage)
Farm groups (ignoring treatments)	6	174·824
Farms within groups	29	38·856***
Linear regression on number of milkings	1	83·087
Residual	28	37·276***
Treatments (eliminating farms)	6	352·892***
Error	66	7·048

*** $P<0·001$.

Table 4*a*. *Experiment D*

Source of variation	Fat content D.F.	Fat content Mean square	Ozone cracking marks D.F.	Ozone cracking marks Mean square
Farm groups (ignoring treatments)	6	7·458	6	1644·99
Farms within groups	25	5·954***	21	1064·23***
Linear regression on number of milkings	1	20·945	1	978·69
Residual	24	5·330***	20	1068·51***
Treatments (eliminating farms)	6	21·031***	6	966·08**
Error	58	0·919	50	253·19

Source of variation	Degrees of freedom	Mean squares, percentages holed before: 30 flexings	1000 flexings	2000 flexings	3000 flexings
Farm groups (ignoring treatments)	6	546·57	115·52	201·85	108·99
Treatments (eliminating farms)	6	38·27	164·32	447·37	227·72
Error	8	154·98	54·95	249·12	154·82

** $P<0·01$; *** $P<0·001$.

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582. IMMERSION CLEANING OF MILKING EQUIPMENT

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with technical assistance from

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(With 2 Plates)

In spite of intensive exhortation of dairy farmers much milk is still of poor bacteriological quality because the milking equipment is inadequately cleaned and sterilized. It has been shown by Akam (1) that the cost of cleaning and sterilizing the milking equipment by the usual methods on farms with about fifteen cows varies from 1*d.* to 3*d.* per gallon of milk produced. Such high costs demand first-class results. Labour was the largest single item of cost so that the reason for indifferent results must be sought elsewhere than in the time spent in washing and sterilizing the milking equipment. Existing methods require attention to detail and possibly present failings are due to lack of attention to a routine job. Obviously the more numerous the manipulations and the items to be cleaned, the greater is the likelihood of slipshod work.

In seeking a method of cleaning less likely to be affected by the human factor, we have simplified the problem by reducing the milk-contact surfaces to the bare minimum for effective milking. Direct-to-can milking is now established in this country, and with in-can cooling it is possible to reduce the surfaces in contact with milk to a few components of small bulk. The few essential milking machine components which remain may be completely immersed for the whole period between milkings in a cleaning fluid with bacteriostatic properties.

Complete immersion of the whole of the equipment between milkings in a bactericidal fluid was advocated in 1925 by Macy (2), but the equipment was washed before immersion. Johns (3), in 1933, described the now well-known system of cleaning milking clusters by keeping them filled with 0·5% caustic soda solution between milkings: he found that caustic soda used in this way could replace both the washing and the sterilizing processes. Since then much information on the worth of this method has accumulated (e.g. Johns (4)). Reid (5), in 1931, showed that liners could be effectively defatted by soaking in a 15% solution of caustic soda overnight, followed by boiling in water for an hour. He also reported that boiling for ten periods each of 10 hr. in 2% caustic soda solution caused only slight softening of the rubber and a slight loss of tensile strength. It was recently shown by Gardner & Berridge (6) that a 7½% solution of caustic soda used at room temperature substantially defatted liners in 7–10 days. This method of dealing with the most serious cause of deterioration of natural-rubber liners is now officially

advocated (7), two sets of liners being used during alternate fortnights, the resting set being immersed in 5% caustic soda solution.

A suitable liquid for immersion cleaning of milking equipment should (*a*) remove milk residues; (*b*) prevent bacterial proliferation; (*c*) limit fat accumulation in natural rubber; and (*d*) not show unfavourable side reactions such as the precipitation of insoluble inorganic salts or calcium soap.

A preliminary trial of immersion cleaning was made at this Institute during July, August and September of 1953. The bacteriological quality of milk produced in a 2-unit direct-to-can parlour, the equipment being cleaned by immersion in 5% caustic soda solution, was equal to that milked with bucket units washed in a hot detergent solution and steamed after every milking. A larger trial was then undertaken on eight commercial farms using 2- or 3-unit walk-through direct-to-can milking parlours. A description of the equipment used and the results obtained during the first year form the subject of this paper.

DESCRIPTION OF EQUIPMENT AND THE IMMERSION-CLEANING TECHNIQUE

Immersion cleaning is specifically designed for direct-to-can milking equipment, and is only applicable if the metal parts are of stainless steel and so simple in shape that all surfaces are accessible to immersion and rinsing fluids. The commercial use of the process is therefore dependent on the specialized components being manufactured, and these could probably be economically introduced on to a farm only when reorganization of the milking arrangements becomes necessary.

The milking machine

A general view of a 2-unit walk-through direct-to-can milking parlour, typical of those now in use in Britain, is given in Pl. 1*a*. The milk can is suspended from a balance (here the can is raised by a vacuum lift), the spring balance having a resetting dial so that both the yield of individual cows and the cumulative weight of milk in the can are known. The balance shows exactly when stripping should take place and when stripping is completed. In this respect the system as a whole is probably better than any other at present in use, except the similar jar-recorder.

The parts of the machine which come into contact with milk, some of which are commercial components and some of our own design, consist of a milking cluster, a can lid with gasket, and two interchangeable lengths of rubber tubing, one used as a long milk tube, the other to connect the can lid to a source of vacuum (Pl. 1*b*). For immersion cleaning it is desirable that all metal parts are of stainless steel to withstand constant immersion in caustic soda solution: this in itself is no deterrent to the use of the process, as stainless steel on its own merits is steadily replacing plated brass. The clusters at present in experimental use are made from liners, shells and clawpieces manufactured by Alfa-Laval Co. Ltd. and Gascoignes (Reading) Ltd. The lid is of our own design and consists of a simple disk of stainless steel plate through which are welded two tubes, one vertical and the other inclined at an angle to direct incoming milk on to the wall of the can, to avoid churning. On the underside of the lid are three studs which hold a gasket loosely so that cleaning solution and rinse water can reach all surfaces (Pl. 1*c*).

In attempting to keep the surfaces as plain as possible for ease of cleaning, several components of normal milking equipment have been omitted; a milk strainer, a shut-off cock

in the milk tube from the claw to the lid, and a vapour trap in the vacuum line from the lid. The strainer serves no obvious useful function in a direct-to-can milking system except to show the presence of clots resulting from mastitis infections. Where the milk from a series of cows is being bulked at milking time a strip-cup should always be used before milking. When milking takes place in a parlour, a cock in the milk line is used to shut off air-flow through the cluster at the beginning of milking, when removing a cluster from a cow, and in a minor emergency. If the long milk tube is kinked to help to remove the cluster from a cow's teats there is no need to use a cock during normal milking as the cluster is either milking or being held in the hand in the cut-off position while being transferred to the next cow. For use at the beginning of milking and in an emergency a special peg is provided on the stalls so that the clawpiece may be hung with the cups in the cut-off position (Pl. 1*a*). A cock is also used when clusters are dipped in disinfectant between milking successive cows as a mastitis control measure. The value of this practice is not well established and it is certainly not universally followed. We have, therefore, not taken account of it. The vapour trap is a component of doubtful value and has therefore been omitted. The rubber vacuum tube is interchangeable with the long milk tube and both are cleaned after each milking.

The immersion cleaning equipment

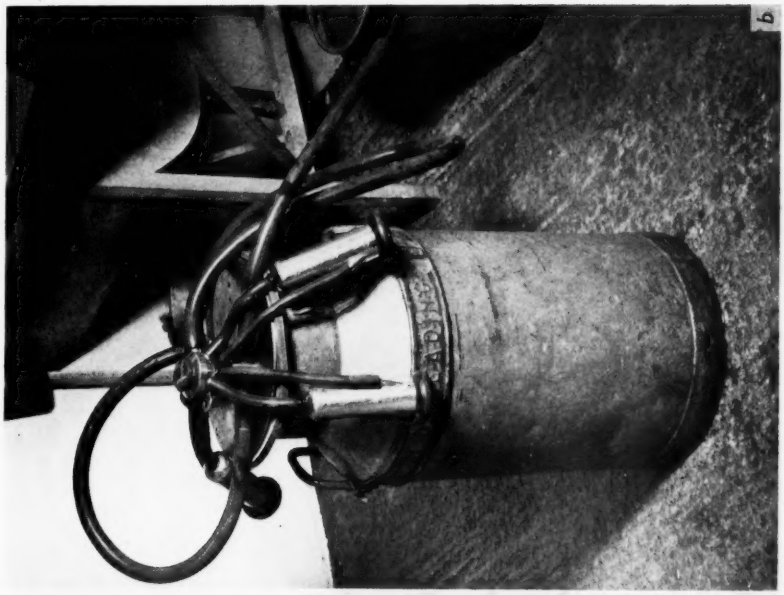
The basket shown in Pl. 2*a* has been designed to hold the milking equipment from a 2- or 3-unit parlour. It has sheet metal walls, a perforated bottom, and handles for lowering it into the cleaning solution. The rubber tubes are retained by a rising spiral of pegs so that air-locking is avoided. The length of the tubes determines the diameter of the basket because it is difficult to place the tubes in position if they extend through more than one complete circle. The clusters are lodged on a bridge. If 3 units are in use, one of the lids is first placed on the mesh floor and the others as shown in the illustration.

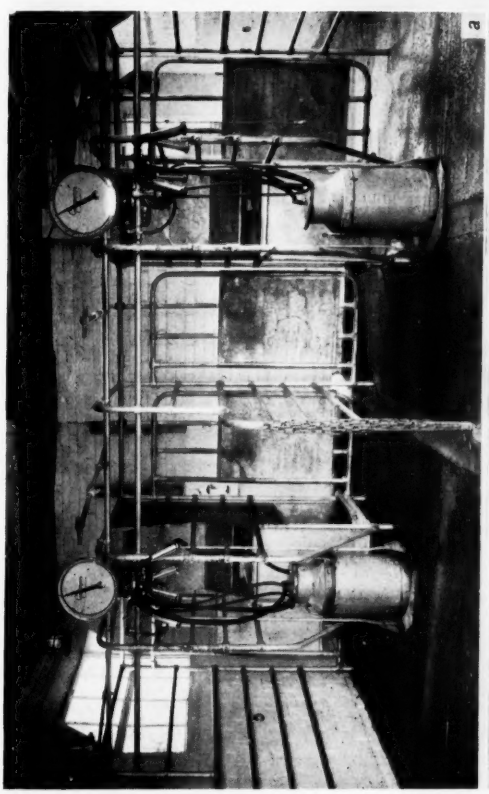
Pl. 2*a* shows that the liners have been released from the teat cups which were then slid a short distance up the milk stems. It was found when the pulsation chambers of the teat cup assemblies were left closed, that sooner or later caustic soda solution penetrated into them; and the only safe procedure was to open these chambers so that the caustic solution and the subsequent rinsing water could enter freely. The shells are retained clear of the liners by lugs on the milk stems (Pl. 2*b*). We are greatly indebted to the Avon India Rubber Co. Ltd., Melksham, Alfa-Laval Co. Ltd., Cwmbran, and Gascoignes (Reading) Ltd., for supplying milk stems with lugs.

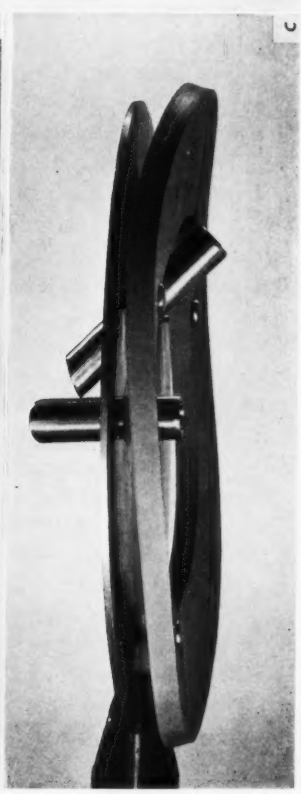
The immersion tank (Pl. 2*c*) holds about 10 gal. of caustic soda solution when filled to the working level which is about 1½ in. above the rim of the basket. The tank has a lid and draincock.

Both the basket and tank are made from black mild steel and are welded throughout. The basket and the inside of the tank remain free from corrosion because of the protective effect of caustic soda. The outside of the tank and lid were treated with chlorinated rubber paint. This finish seems to be satisfactory if renewed at yearly intervals, but vitreous enamel might be even more satisfactory.

A small rinse trough and a working surface about 2 ft. × 18 in., covered with sheet rubber to prevent damage to the metal components, complete the cleaning equipment (Pl. 2*c*). There should, of course, be a supply of hot water in the dairy to take the chill off







THIEL, CLOUGH & CLEGG—Immersion cleaning of milking equipment

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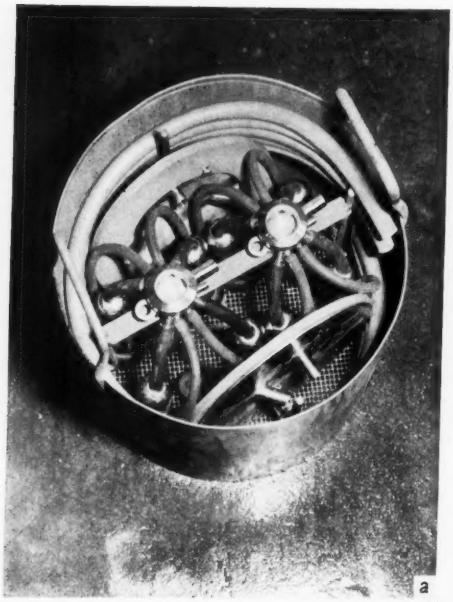

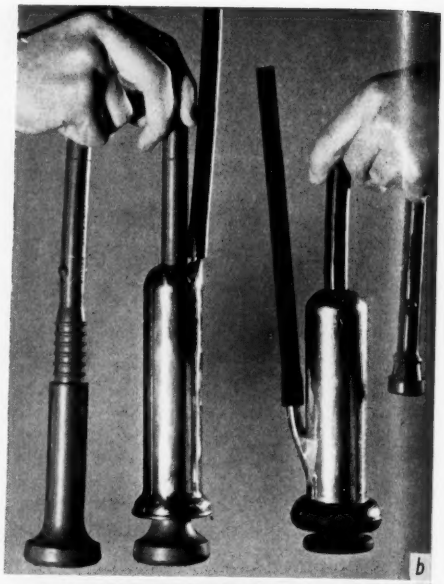

THIEL, CLOUGH & CLEGG—IMMERSION CLEANING OF MILKING EQUIPMENT

rinse water and udder-washing water in winter time, for making up calf food, washing calf-feeding buckets and cleaning any dirty milk cans. A supply of dairy detergent and approved hypochlorite would also be required.

Technique of immersion cleaning

The treatment of the equipment is the same at each milking. The basket containing the gear is lifted clear of the caustic soda solution and lodged on the rim of the immersion tank to drain. A few gallons of water are then run into the rinse trough and hypochlorite added. The basket of gear is then placed on the floor and the contents sprayed with water from a hose to remove external caustic soda so that the equipment may be safely transferred by hand to the rinse trough. It is then convenient to rinse the long rubber tubes by holding their ends one at a time to the tap. The other components are rinsed free from caustic soda by lifting them in and out of the rinse water, the clusters are then spread-eagled one at a time on the working surface, the liners drawn on to tension, and the long rubber tubes are attached to the clawpiece and lid. The whole unit is then placed on a milk can ready for transfer to the parlour (Pl. 1*b*).

After milking, the units are placed in the trough which still contains the water used before milking, the long rubber tubes are detached and placed in the basket, and the liners released from the shells which are then slid over the retaining lugs on the milk stems. The clusters are placed on the bridge in the basket, the lids carefully placed alongside them so that none of the shells is pushed over the retaining lugs, and the basket lowered into the immersion tank.

The operations of preparing the equipment for milking and returning it to the immersion tank take about 5 min. per cluster per milking. Some care is required in rinsing before milking or trouble with slipping rubberware will occur. This is a useful safeguard against inadequate rinsing. As the rinsing that occurs as a result of demounting the liners in the trough is adequate, no particular care was taken with rinsing after milking. The equipment was completely dismantled after each period of 4 weeks to remove accumulated deposits and to inspect the rubberware. At the same time the caustic soda solution was renewed.

Milk cooling

If milk is received direct from the cow into the final shipping can, obviously cooling should take place in this can. Without increasing the articles needing cleaning, this may be done by applying water to the outside of the can by immersion or spraying. When the water is refrigerated either of these methods will give fast cooling, but where ordinary tap water is used the milk will take 1½–2 hr. to cool, and 6–10 times its volume of water will be used. If these methods are not practicable a turbine cooler is probably the best alternative. The agitator may be adequately cleaned by washing in a detergent solution containing hypochlorite. After the milking equipment has been removed to the immersion tank, some detergent is added to the chlorinated rinse water already in the trough and the turbine cooler washed. This method has been used successfully for a year on several commercial farms where immersion cleaning has been in experimental use.

The photographs shown in Pls. 1*a*, 2*c* and *d*, were taken at the farm of S. Spencer Gates, Esq., Barkham, Berkshire. At this farm we were able to lay out the dairy at the time it

was built to suit immersion cleaning. No heating equipment was installed because an abundant supply of hot water was available in the house nearby. An adequate supply of cooling water was available from a gravity-fed piped supply and the milk was not collected until midday. An immersion cooling tank was therefore built with one removable wall and a self-operating device for shutting off the water supply after a pre-determined interval (Pl. 2*d*).

IMMERSION CLEANING TRIALS ON COMMERCIAL FARMS, 1954

To provide information on the suitability of immersion cleaning under practical conditions eight farms were supplied during April and May 1954 with the equipment described above. As the method is primarily designed for use with direct-to-can milking equipment, farms using 2 or 3 such units in walk-through parlours were selected. This type of parlour is still comparatively rare, so that no criterion other than nearness to Reading was needed in making the selection. Because most of these farms were equipped with Alfa-Laval milking machines and in most cases Alfa-Laval stretched liners (No. 20003B), all experimental work reported here was done with clusters based on this liner. The mouthpiece and grab rings used were specially designed to hold to the rubber in spite of the lubricating effect of caustic soda.

With such a small number of farms general information on the process of immersion cleaning could be obtained only by strictly limiting the number of experimental procedures. In fact the equipment and the cleaning process were identical on all farms, with the single exception that four farms used 1% caustic soda solution in the immersion tanks and the other four farms used a 5% solution.

It was known from the preliminary experiment that plain caustic soda solution caused deposits in the liners. These deposits, which consisted in part at least of calcium soap, could be removed by soaking in dilute acid followed by brushing with alkaline detergent. As no method of avoiding deposits had been tried it was decided in the first instance to remove precipitates once a month with phosphoric acid at the same time as liners were cut and reversed. The full treatments of the milking equipment in the monthly cycle were as follows:

At each milking, farms using 5% *caustic soda solution.* Immediately before milking the basket and gear were removed from the caustic solution, drained, and rinsed in a water-rinse tank. The gear was then removed to a wash trough containing 10 gal. of tap water with 1 oz. of hypochlorite. After final rinsing the equipment was assembled and milking begun. At the end of milking the milking units were returned to the wash trough, rinsed in the same hypochlorite solution, demounted, packed into the basket and returned to the caustic soda solution.

At each milking, farms using 1% *caustic soda solution.* The procedure here was similar except no water-rinse tank was used, so the equipment was transferred direct from caustic soda solution to the chlorinated rinse.

At monthly intervals, all farms. At the morning milking the caustic soda solution was rejected and the tank filled with ½% phosphoric acid solution, in which the milking equipment remained until the afternoon. It was then completely dismantled, brushed in alkaline detergent solution, and the liners reversed and cut to length. A new solution of caustic soda was made up, and at the farms using 5% caustic soda solution the water in the rinse tanks was renewed.

SAMPLING AND TESTS

A milk sample was taken weekly from the first can filled by one of the units at morning milking, iced, and tested within 3 hr. Variable contamination from the can was avoided by taking a clean can the evening before and leaving it overnight completely filled with water to which ½ oz. of hypochlorite solution had been added. To measure the bacteriological state of the milking equipment clusters were rinsed once a week by passing 500 ml. of ¼ strength Ringer's solution through the cluster 5 times. A weekly sample of caustic soda solution and first rinse water were also taken.

Extensive bacteriological tests were made on the milk samples but reference will only be made to the following: keeping quality and initial colony count as measures of general milk quality, and initial coli-aerogenes presumptive tests to give a measure of the cleanliness of the milking technique. Keeping quality was determined by tests every 3 hr. (after the first 24 hr.) with the milk incubated continuously at 22° C. to clot-on-boiling end-point. Presumptive coli-aerogenes tests were made in MacConkey's broth and the colony counts of milk, caustic soda solution, water rinse and Ringer's solution were determined by plating in Yeastrel milk agar. Incubation for these three tests was at 30° C. for 3 days.

More detailed bacteriological work in relation to immersion cleaning is reported elsewhere (8).

RESULTS AND DISCUSSION

Bacteriological quality of milk

Regular examinations of milk samples were made to determine whether the equipment was suitable for milking, as well as to show the effectiveness of immersion cleaning. The instructions for cleaning the equipment were strictly followed and end-of-month treatments (soaking in acid and cleaning, cutting and reversing liners) were carried out by N.I.R.D. staff. No attempt was made to influence milking technique, including udder washing, although advice was freely given when asked for. It is likely, however, that frequent visits would increase the care taken, leading to milk of improved quality.

The results given in Table 1 are typical of those obtained throughout the experimental period. They were no doubt influenced by the abnormally cool summer. The figures for keeping quality of milk samples were entirely satisfactory, a value at 22° C. of 36 hr. being good and 42 hr. excellent. Colony counts per ml. of milk are also generally good, 5000 being considered a good standard under normal working conditions. The coli-aerogenes figures do not, however, indicate a uniformly high level of cowshed technique, the absence of these organisms in 0·1 ml. of milk being taken as no more than satisfactory. The results from the rinse samples are discussed later.

It may also be seen from Table 1 that milk samples from farms using 5% caustic soda solution are not obviously different from those using 1%. Nor is there any significant difference between samples taken at the beginning and the end of the monthly cycles. There may, however, be a slight difference in favour of samples obtained with new liners compared with liners which had already been used for 2–3 months.

From the examinations of milk samples it seems that under the conditions of operation on these farms satisfactory milk was produced with the equipment cleaned by the immersion process.

Table 1. *Selected bacteriological data*

(Bacteriological results for periods of 4 consecutive weeks at various stages of liner life.)

Farm	Caustic soda, nominal per-centage		Old liners, July–Aug.				New liners, Aug.–Sept.				Old liners, Oct.–Nov.			
			13–16 weeks				1–4 weeks				9–12 weeks			
A	5	Keeping quality (hr.)	54	54	54	57	54	54	60	45	54	54	54	—
		Count (thousands/ml.)	2·7	2·2	3·6	3·8	1·5	2·1	2·6	1·5	2·0	3·0	1·9	—
		Coli, absent in (ml.)	1	0·1	1	1	1	0·1	1	1	0·1	1	0·1	—
		Rinse, thousands per cluster	1100	50	—	1000	0·5	2	16	1	31	1	250	—
			13–16 weeks				1–4 weeks				13–16 weeks			
B	5	Keeping quality (hr.)	48	45	48	51	51	51	54	57	54	54	45	48
		Count (thousands/ml.)	1·1	2·4	4·0	0·7	1·1	0·6	1·1	5·0	1·3	3·3	2·5	4·1
		Coli, absent in (ml.)	1·1	0·001	0·0001	0·1	0·1	0·1	1	1	0·1	1	0·1	0·01
		Rinse, thousands per cluster	—	500	—	300	0·5	0·5	40	0·5	4	1	18	300
			9–12 weeks				1–4 weeks				13–16 weeks			
C	5	Keeping quality (hr.)	42	—	45	51	51	—	54	54	54	54	54	54
		Count (thousands/ml.)	17·6	—	9·2	16	16·6	—	4·2	3·2	4·1	2·0	2·5	3·0
		Coli, absent in (ml.)	0·0001	—	0·001	0·1	0·001	—	0·1	0·01	1	0·1	0·1	0·1
		Rinse, thousands per cluster	—	—	75	1	75	0	4	7	2	10	3	50
			9–12 weeks				1–4 weeks				13–16 weeks			
D	5	Keeping quality (hr.)	48	—	54	51	54	—	48	39	48	48	51	39
		Count (thousands/ml.)	10·7	—	1·2	7·8	31·9	—	3·7	3·5	3·1	0·8	3·0	1·4
		Coli, absent in (ml.)	1	—	0·001	0·001	0·1	—	0·001	0·01	0·01	0·1	0·01	0·01
		Rinse, thousands per cluster	—	—	500	15	80	0	0	0·5	10	2000	20	10
			9–12 weeks				1–4 weeks				9–12 weeks			
E	1	Keeping quality (hr.)	54	—	57	54	57	—	54	54	51	51	54	54
		Count (thousands/ml.)	0·3	—	3	2·5	1	—	0·7	1·4	0·1	0·2	21·0	1·0
		Coli, absent in (ml.)	1	—	1	0·1	0·1	—	1	0·1	1	1	0·01	0·01
		Rinse, thousands per cluster	—	—	2500	—	34	1	0	0·5	0·5	0	0	0
			9–12 weeks				1–4 weeks				13–16 weeks			
F	1	Keeping quality (hr.)	45	42	45	54	45	—	45	48	54	51	51	51
		Count (thousands/ml.)	2·9	2·4	4·0	2·0	0·5	—	0·1	0·2	2·1	1·1	1·0	0·5
		Coli, absent in (ml.)	1	1	0·1	1	0·01	1	1	1	0·1	0·01	0·01	0·1
		Rinse, thousands per cluster	—	1000	3500	—	80	30	220	0	130	19	73	55
			9–12 weeks				1–4 weeks				9–12 weeks			
G	1	Keeping quality (hr.)	54	54	—	48	51	42	60	51	54	54	48	51
		Count (thousands/ml.)	1·6	6·7	—	3·6	0·7	0·3	1·3	0·1	0·8	1·3	1·2	2·3
		Coli, absent in (ml.)	0·1	0·01	—	0·01	0·1	0·01	1	1	1	0·1	0·01	0·01
		Rinse, thousands per cluster	1500	500	1000	500	—	2	0·5	0·5	0·5	6·5	17	12
			9–12 weeks				1–4 weeks				9–12 weeks			
H	1	Keeping quality (hr.)	—	54	42	42	60	54	54	54	45	54	51	54
		Count (thousands/ml.)	—	7·5	2·6	6·1	3·0	1·5	0·6	1·5	2·4	1·3	3·4	2·5
		Coli, absent in (ml.)	—	0·001	0·0001	0·001	0·1	0·1	1	0·1	0·01	1	1	0·01
		Rinse, thousands per cluster	500	5	50	7	0	0·5	0·5	2	12	150	1·5	3

Dashes in the table indicate no tests were made.

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Bactericidal properties of the immersion solutions

In Table 2 bacterial counts are given of the immersion solutions on two typical farms, one using 5% and the other 1% caustic soda solution. Alkalinities, as titratable NaOH, are also given. From the beginning to the end of the monthly cycles the caustic soda strength fell to about two-thirds of the original value. On the farms using 5% solution where a water-rinse tank was in use, the rinse solution started as plain water and gained in caustic soda to a maximum of 0·5–1% as NaOH. Bacterial counts remained low in the nominal 5% caustic soda solutions, but were higher in the water rinse on these farms (but are lower than the average farm well or spring water supply), and were also higher in the nominal 1% caustic solutions on the other farms. As a result of this experience it has been decided to use an intermediate strength solution of caustic soda in future work and no first rinse tank will be used. Caustic soda on the outer surfaces of the equipment will be removed by hosing before transferring the components into the rinse trough.

Atmospheric shade temperatures for the days on which samples were taken are also shown in Table 2. Generally speaking no marked difficulty is experienced with milk quality unless the AST figures are above 67° F., so that no doubt the bacterial counts would have been higher on occasions in a warm summer.

Bacteriological state of teat cup liners

It was first thought that a low bacterial count of the immersion solution indicated a low bacterial population on the immersed equipment. It was later found by rinsing clusters with sterile Ringer's solution that this was not so. The numbers of organisms which could

Table 2. *Colony counts per ml. and alkalinities of caustic soda solutions and first rinse solutions*

	Farm C, 5% caustic				Farm F, 1% caustic.		
	Caustic tank		Rinse tank		Caustic tank		
Date	% NaOH	Count/ml.	% NaOH	Count/ml.	% NaOH	Count/ml.	A.S.T.* (° F.)
27 June	—	0	—	0	—	6	64·0
11 July	—	1	—	10	—	22	67·5
25 July	3·96	5	0·18	3	1·34	50	64·5
8 Aug.	3·36	9	0·51	16	1·15	31	59·5
15 Aug.	3·16	3	0·65	121	1·06	171	62·0
22 Aug.	5·44	0	0·20	20	1·52	96	59·5
29 Aug.	5·20	1	0·42	78	1·32	30	64·0
5 Sept.	3·60	1	0·47	5	1·04	10	61·0
12 Sept.	3·28	2	0·58	6	0·98	19	58·0
19 Sept.	4·76	1	0·15	1	0·98	1	56·0
26 Sept.	4·48	2	0·34	3	0·93	17	55·5
3 Oct.	4·20	0	0·51	4	0·84	7	52·0

* A.S.T. = atmospheric shade temperature = mean of the average of maximum and minimum atmospheric shade temperatures at both 9 a.m. and 5 p.m.

be rinsed off clusters are shown in Table 1, from which it may be seen that old liners often showed high counts. Liners in the first month of use seldom showed counts above 50,000 per cluster. The clusters were rinsed 5 times and the plates incubated at 30° C. A count of 50,000 per cluster with this technique approximates to the 'satisfactory' standard of 5000 obtained by rinsing the clusters only once and incubating the plates at 37° C.

In spite of the fact that good quality milk was produced with the equipment, the process of immersion cleaning cannot, in view of the results for cluster rinses, be considered completely effective in its present form. Because the immersion solutions themselves showed only low counts it seems that bacteria on the liners are protected from the caustic soda, and grow either in or on the rubber surfaces or in the deposited material on those surfaces. Old liners showed much higher counts than new liners. Improved bactericidal action at the rubber surface is essential to the complete success of the process, and this may be possible if the occurrence of protective films can be avoided: alternatively, the presence of a bactericide in the rubber may be effective. Future work will be along these lines.

Liner life

In the initial stages of this experiment liners were kept in continuous use to give some indication of the expected life under the variety of conditions on the eight experimental farms. However, to suit the bacteriological work it was convenient to change all liners on a given date. At this stage many of the original liners were still satisfactory, while on three farms the replacement rate was high or very high (Table 3). On most of the farms the duty was high but there was a considerable increase in the life of liners compared with the farmers' previous experience.

Table 3. *Data on teat cup liners used for* 12 *weeks*

Farm	Nominal size of milking herd and breed	No. of liners in use	No. of liners replaced and causes
A	35 Jersey	8	2, slipping mouth rings
B	40 Ayrshire	12	2, mechanical damage
C	30 Guernsey	8	3, fatigue splitting
D	35 Ayrshire	8	1, fatigue splitting
E	12 Shorthorn	8	1, mechanical damage
F	18 Ayrshire 22 Jersey	8	3, manufacturer's fault 1, mechanical damage 8, fatigue
G	40 Ayrshire	12	Nil
H	18 Guernsey	8	Nil

As a result of reversing liners at monthly intervals and the defatting action of the caustic soda, the mouths of liners remained in satisfactory condition, no liners being rejected because of loss of shape at the mouth end. However, in spite of the use of rings which gripped the rubber much more tenaciously than standard equipment, difficulties due to slipping have been experienced. This occurred particularly on Farm F, but also on Farm C. On farms where slipping occurred the liners became thickened and hard, and finally failed because of splitting of the barrel (noted in Table 3 as 'fatigue splitting'). It is thought that this condition is associated with water hardness, leading to deposition of a rigid layer of material. Flexing of the rubber then causes this layer to break into elongated plates resulting in stress concentration at the joint lines of adjacent plates. The total hardness of the water supplies on these farms varied from about 150 to 300 p.p.m.

Metal ware

Stainless steel teat-cups and can lids remained in good condition. Plated clawpieces, used in the absence of stainless steel ones, lost most of the plating in the bores of the milk connexions but otherwise withstood immersion cleaning well. The basket used to contain

the equipment during cleaning and the interiors of the immersion tanks remained in good condition. Exterior painting of these tanks with bitumastic paint was not satisfactory, but painting once a year with chlorinated rubber paint is satisfactory.

SUMMARY

A method of cleaning milking equipment is described which involves the immersion of all items coming into contact with milk in a caustic soda solution for the whole period between milkings. It is expected that this method of maintaining the equipment in satisfactory bacteriological condition would need less attention to detail than existing techniques. The procedure outlined may only be applied to apparatus which can be packed into a basket no larger than can be readily handled. It therefore applies only to direct-to-can milking equipment, and suitable milking and immersion cleaning apparatus are described.

The process has been tried for a year on 8 commercial farms. The results are promising in that milk of good quality has been consistently produced, but the bacteriological state of the milking clusters was often below present standards. Deposition of calcium soap and inorganic calcium salts was marked on farms where the total hardness of the water was of the order of 200 p.p.m. Work is continuing.

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REFERENCES

(1) AKAM, D. N. (1955). *J. Soc. Dairy Tech.* **8**, 94.
(2) MACY, H. (1925). *Circ. Minn. Coll. Agric. Ext.* no. 21.
(3) JOHNS, C. K. (1933). *Sci. Agric.* **13**, 460.
(4) JOHNS, C. K. (1943). *J. Milk Tech.* **6**, 274.
(5) REID, J. F. (1931). *India Rubb. J.* **82**, 563.
(6) GARDNER, E. R. & BERRIDGE, N. J. (1952). *J. Dairy Res.* **19**, 31.
(7) MINISTRY OF AGRICULTURE (1954). *Advisory Leaflet*, no. 422. London: H.M.S.O.
(8) CARREIRA, D. F. C., CLEGG, L. F. L., CLOUGH, P. A. & THIEL, C. C. (1955). *J. Dairy Res.* **22**, 166.

EXPLANATION OF PLATES

PLATE 1

(*a*) A 2-unit walk-through parlour with food hoppers. Note peg for holding clusters in the cut-off positions.
(*b*) Milking equipment ready for transfer to the parlour.
(*c*) Can lid showing gasket loosely retained by studs.

PLATE 2

(*a*) Milking equipment in the basket.
(*b*) Liners in the cleaning position inside teat cups, and showing lugs on the stems.
(*c*) Immersion tank, rinse trough and rubber-covered table.
(*d*) A can-immersion cooler.

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583. THE EFFECT OF STEAM, HYPOCHLORITE AND CAUSTIC SODA USED FOR TREATING DIRECT-TO-CAN MILKING EQUIPMENT, ON THE BACTERIOLOGICAL QUALITY AND FLORA OF MILK

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(With 1 Figure)

The rubber surfaces of a milking machine that come into contact with milk (i.e. the teat cup liners and long milk tubes) are the most difficult parts to clean and sterilize. The reason for this is that fat from milk and the surface of the udder promotes oxidation (1) which so affects the rubber surface that it can only be cleaned adequately by materials which penetrate. Heat penetrates and sterilizes but does not clean rubber. Detergents and disinfectants which act at the surface are effective only with prolonged contact. This led Johns (2) to recommend wet-storage of teat cup liners in 0·5% caustic soda. This method, or an alternative, using 2% alkaline dairy detergents plus 125 p.p.m. sodium hypochlorite, is now recommended in Advisory Leaflet 422 (3).

The use of 5% caustic soda at room temperature proved to be satisfactory in defatting rubber (4, 1). The solution is also strongly bactericidal (5).

A preliminary trial at this Institute (1953) of direct-to-can milking with the complete milking equipment (with stainless steel metal parts) cleaned by immersion in 5% cold caustic soda between milkings, gave excellent bacteriological results. A trial on eight commercial farms was made during 1954, and a detailed report of the process now known as immersion cleaning is described by Thiel, Clough & Clegg (6).

As this method of cleaning and sterilizing yielded milk of excellent keeping quality in a small trial, it was decided to find out whether the bacterial flora of the milk was different from that produced by equipment cleaned by brushing in a hot detergent solution and sterilized by steam or sodium hypochlorite, and also to examine the bacteriological quality of the milk. These two aspects of this work are reported in separate sections.

BACTERIOLOGICAL QUALITY OF MILK

Experimental methods

Technique of immersion cleaning

Immersion cleaning is a method of cleaning the milking equipment associated with direct-to-can milking. All the milk-contact surfaces except the can are either of stainless

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steel or rubber, and are completely immersed in caustic soda between milkings. The only articles which are in contact with milk during milking are the teat cup cluster with stainless steel shells and claw piece, the long milk tube and the can fitted with a specially designed stainless steel lid and rubber gasket. In this series of experiments these utensils, together with the stall tubes, were placed in a mild steel basket fitted with two handles to lower it into a mild steel container filled with 5% caustic soda. Before milking the basket was removed from the caustic soda container, allowed to drain, and transferred to a plain water container to rinse off the greater part of the caustic soda. The milking parts were then removed by hand and immersed in water plus chlorine (60 p.p.m.) to complete the rinsing without contamination. After milking the clusters were rinsed in the same chlorinated water, the rubbers demounted inside the shells, to allow subsequent rinsing free from caustic soda, and together with the lids were fitted into the basket and again immersed in caustic soda until the next milking. (For a more detailed description of the process see pp. 158–159 of this number of the *J. Dairy Res.*)

Both the caustic soda solution and the water in the preliminary rinse tank were renewed at monthly intervals.

Farm methods

Two farms were selected for the experiment, which covered the period February–September 1954. Farm A was a small farm with an outside bail where the utensils were treated with sodium hypochlorite. Farm B was a larger farm with a milking parlour where the utensils were sterilized by steam. On both farms the experimental treatment alternated with the control treatments for successive periods of 2 months. While the experimental treatment was in use at one farm a control treatment was used at the other. The rubberware coming in contact with milk was renewed at the beginning of each period of 2 months on both farms. The following are the methods of utensil treatment at the farms.

Farm A. After the morning milking the equipment was rinsed in cold water and brushed in a hot detergent chlorine wash. After the evening milking the equipment received only a plain water rinse followed by a cold rinse in chlorinated water, as recommended in *Advisory Leaflet*, no. 422.

Farm B. After each milking the equipment was rinsed in cold water and washed in hot detergent solution. The clusters were steamed on jets and the rest of the equipment in a steam chest.

At both farms sodium hypochlorite was used in the udder washing water, but in the first part of the work no special care was taken to see that udder washing was well done.

The treatment with caustic soda was used first on farm B and continued for 8 weeks. At the same time sodium hypochlorite was used as a control on Farm A. At the end of 8 weeks the treatments were changed, with farm B using steam sterilization as control.

Bacteriological tests

So that the results were not influenced by the bacteriological condition of cans returned from the creamery, a can which had been steamed for 30 min. was supplied to each farm on the day of sampling. Once every week a milk sample was taken during the morning milking from each farm, iced and tested within 3–4 hr.

At farm A the first can contained the milk from only 3–4 cows, whereas at farm B it contained the milk from 6–7 cows.

At the same time samples of the 5% caustic soda solution and the liquid in the preliminary rinse tank were taken for bacteriological examination and to check the amount of caustic in the solutions. Periodically, clusters were rinsed 5 times with 500 ml. of ¼ strength Ringer's solution.

The milk samples were divided into two parts; one was tested on the day of sampling, and the other was aged at 22° C. for 24 hr. in a water-bath. Caustic soda and water samples were neutralized before testing (see below).

The following tests were begun between 9.30 and 10.30 a.m.:

Keeping quality. This was determined by incubating the milk at 22° C. and after 24 hr. examining every 3 hr. for the clot-on-boiling end-point.

Colony count. Plates were poured in triplicate with Yeastrel milk agar (Y.M.A.) and incubated at 30° C. for 3 days.

Total spore counts. Approximately 10 ml. of milk, caustic soda or rinse solution (previously neutralized) were heated at 80° C. for 10 min., cooled, and 1 ml. quantities plated with Y.M.A. and incubated at 30° C. for 3 days.

Resistant spore count. Quantities of 50, 5 and 0·5 ml. were heated for 30 min. at 100° C., cooled and incubated for 14 days at 30° C. before examination to determine the most probable number. The smallest quantity was diluted in 10 ml. autoclaved litmus milk. Those tubes which showed no change were tested by the alcohol test for sterilized milk (80% alcohol containing 0·02% neutral red).

Coli-aerogenes test. Quantities of 10, 1 and 10^{-1} ml. of milk were inoculated into MacConkey's broth and incubated at 30° C. for 3 days. For the aged samples 10^{-2}, 10^{-3} and 10^{-4} ml. dilutions were used. When using 10 ml. quantities, double-strength MacConkey's broth was used.

Results

The results from the two farms are given separately for comparison. Fig. 1 shows that the differences between the colony counts during the different periods are small. Differences between treatments were expected to be more apparent with aged samples, but no appreciable differences were found. During the fourth period on both farms, i.e. with both caustic and steam treatments, there was an increase in the number of samples with high initial counts. This difference may have been caused by some factor outside the experimental technique, probably the season. While in both initial and aged samples there are only small differences between control and caustic treatments, these differences are in favour of the caustic treatment on all but one occasion.

During the first three 8-week periods plates for colony counts were also incubated at 22° C. for 5 days with the unaged samples, but as the results at 22 and 30° C. were similar the former test was discontinued. Similar results were also observed with colony counts incubated at 22 and 30° C. with aged samples.

Table 1 shows the variation in numbers of coli-aerogenes throughout the four periods. The counts generally were low, indicating good cowshed technique. The initial count bears no relation to that of the aged milk. While the absence of coli-aerogenes/100 ml. in the fresh milk may mean a similar result in the aged milk, there were instances where initially these organisms were absent in 100 ml. but the aged milk gave high counts.

As it seemed possible that variations in udder washing or the cleaning of the clusters were responsible for variation in results, a rigid technique of udder washing was used for

the fourth period at both farms. Individual samples were taken from those cows whose milk was sampled from the first can; samples were also taken from the udder washing water and from cluster rinses.

The udder washing technique consisted of washing the udders with a solution of 1 oz. sodium hypochlorite to 2 gal. water, wringing out the cloth outside the bucket, washing the udder a second time, followed by wringing out the cloth again outside the bucket, and, finally, wiping the udder dry. Where the udders were visibly dirty they were cleaned with water before using the hypochlorite solution.

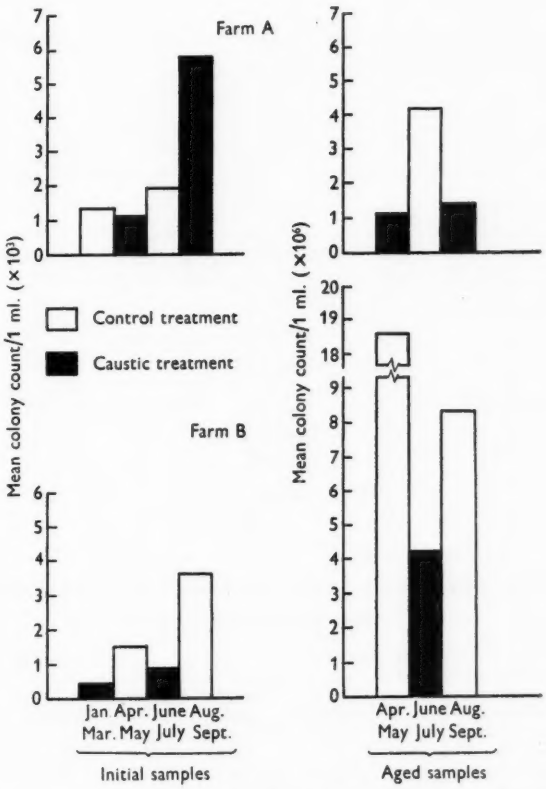


Fig. 1. Mean colony counts at 30° C. of initial and aged milk samples on two farms with control and caustic treatments.

As the bacteriological rinsing of clusters might have influenced the normal cleaning the rinses were not taken from the clusters used to produce the milk which was sampled.

Table 1 shows that this udder washing technique during the last period brought about no apparent reduction in the number of coli-aerogenes organisms in the milk. No coli-aerogenes organisms were found in the udder washing solutions which were neutralized immediately on sampling. Neither were coli-aerogenes organisms found in any of the cluster rinses. In approximately 80 individual cow samples, coli-aerogenes organisms were only determined in three samples, and then only in 10 ml. quantities.

Table 1. *Individual numbers of coli-aerogenes with fresh and aged milk samples from different treatments*

	Most probable number of coli-aerogenes/100 ml., 3 days at 30° C.					
	Farm A			Farm B		
Week	Treatment	Initial	Aged	Treatment	Initial	Aged
2	Hypochlorite	3	—	Caustic soda	7·3	—
3		3·6	—		0	—
4		28	—		0	—
5		23	—		0	—
6		0	—		0	—
7		0	—		0	—
8		0	—		0	—
9	Caustic soda	39	—	Steam	23	—
10		0	—		0	—
11		3·6	—		150	—
12		75	—		0	—
13		0	—		0	—
14		0	0		1,100+	11,000,000
15		0	0		0	930,000
16		0	0		9·1	150,000
17	Hypochlorite	0	0	Caustic soda	3·6	1,100+
18		28	11,000,000		3·6	46,000
19		0	0		43	110,000+
20		15	45,000,000		0	1,100,000+
21		0	0		0	0
22		3·6	150,000		3·6	110,000+
23		3	0		43	110,000+
24		9·1	230,000		7·2	11,000,000+
25	Caustic soda	3·6	0	Steam	19	53
26		0	0		0	0
27		240	11,000,000		0	0
28		23	36,000		0	0
29		0	0		1,100	11,000,000+
30		6·2	21,000		33	1,000,000+
31		0	0		3	1,000,000+
32		0	0		0	110,000+

Table 2. *Keeping quality results (to* C.O.B. *end-point at* 22° C.*) with different treatments*

	Distribution of keeping quality results							
	Farm A				Farm B			
Hr. to C.O.B.	Hypochlorite	Caustic soda	Hypochlorite	Caustic soda	Caustic soda	Steam	Caustic soda	Steam
33	0	0	0	0	1	0	0	0
36	0	0	0	0	0	0	0	0
39	0	0	0	0	0	1	1	2
42	2	0	1	1	3	1	2	1
45	0	1	0	0	1	1	0	0
48	1	1	0	0	0	1	0	0
51	3	4	3	2	1	4	2	4
54	1	2	3	2	1	0	2	0
57	0	0	1	3	0	0	1	1
Mean values/ period (hr.)	48·4	50·6	51·5	52·8	44·1	47·2	48·7	47·8
Mean values/ treatment/ farm (hr.)		49·9	51·7			46·4	47·5	
Average A.S.T./ period (° F.)	44·3	52·6	63·8	62·4	44·3	52·6	63·8	62·4

The distribution of the keeping quality results of the milk from both farms is given in Table 2, together with the mean values for each period and for each treatment. With farm A the caustic soda treatment gave better average keeping quality than the hypochlorite control in both periods, but the means of both periods yield a difference of only 1·8 hr. in favour of the caustic soda. The results from farm B favour the steam control, although the mean difference was only 1·1 hr.

Table 3 shows a remarkable uniformity of total spore counts between samples. Further, no differences can be attributed to differences in treatment. Ageing of the milk samples for 24 hr. has had no effect on numbers except in two cases.

Table 3. *Number of total spores in individual samples in different treatments*

	Colony count/ml. 3 days at 30° C.					
	Farm A			Farm B		
Week	Treatment	Initial	Aged	Treatment	Initial	Aged
2	Hypochlorite	4	—	Caustic soda	20	—
3		10	—		13	—
4		46	—		27	—
5		13	—		6	—
6		20	—		12	—
7		8	—		11	—
8		8	—		8	—
9	Caustic soda	12	—	Steam	1	—
10		18	—		1	—
11		<1	—		1	—
12		1	—		76	—
13		3	—		6	—
14		<1	24		4	28
15		6	11		13	30
16		9	11		14	11
17	Hypochlorite	6	7	Caustic soda	2	6
18		15	14		2	14
19		4	15		5	8
20		3	10		8	16
21		15	12		61	64
22		3	3		4	14
23		2	13		2	5
24		0	4		0	0
25	Caustic soda	0	1	Steam	1	11
26		2	4		1	5
27		*	*		*	*
28		12	7		*	*
29		1	*		2	9
30		1	*		3	30
31		1	2		65	*
32		7	*		*	*

* Results masked by micrococci.

The numbers of resistant spores (Table 4) show a similar picture to that of total spores, except that the incidence of resistant spores is much lower. Here there are no apparent differences between treatments, and ageing of the milk for 24 hr. brought about no change.

Tests on the caustic soda solution and the first rinse water were made each week and total colony count and total number of spores determined. In no case was the total colony count of the caustic solution higher than 100/ml., or of the water higher than 500/ml. The spore counts in the caustic soda solution did not exceed 10/ml. and were usually 1 or 2/ml.,

and in the water were not higher than 50/ml. and usually about 5/ml. There was no relationship between the bacteriological results and the gradual fall in concentration of caustic soda from 5 to 3·5% during the month before the solutions were changed.

Table 4. *Number of resistant spores in individual samples in different treatments*

	Most probable number/100 ml. 14 days at 30° C.					
	Farm A			Farm B		
Week	Treatment	Initial	Aged	Treatment	Initial	Aged
2	Hypochlorite	0	—	Caustic soda	0·36	—
3		2·3	—		0	—
4		0·91	—		0·91	—
5		2·3	—		2·3	—
6		0	—		0·36	—
7		0	—		0·36	—
8		0·36	—		0	—
9	Caustic soda	0	—	Steam	0	—
10		0	—		0	—
11		0·36	—		0	—
12		0	—		0	—
13		0·36	—		0	—
14		0	0		0	24
15		0	0		0	0
16		0	0		0	0·6
17	Hypochlorite	0	0	Caustic soda	0	0
18		0	4·6		0	4·6
19		0	0		0	0
20		0	0·72		0·72	0·72
21		0·72	5·8		0·6	4·6
22		0	1·88		0	3·2
23		48	5·8		0	0
24		4·6	4·6		0	4·6
25	Caustic soda	48	3	Steam	1·46	0
26		3·0	0		0	0
27		8·6	4·6		0	0
28		0	0		0	0
29		0	3·1		1·82	120
30		0	0		0	0
31		49	19·6		49	49
32		0	0		0	0

DETERMINATION OF MAIN BACTERIAL TYPES

The main types of micro-organisms occurring in all the milk samples in this investigation were examined to find if there were differences between the caustic soda and control treatments. The observations included the flora of the milk when fresh and when aged for 24 hr. at 22° C. This ageing was chosen as representing the worst condition under which milk could be received at the creamery.

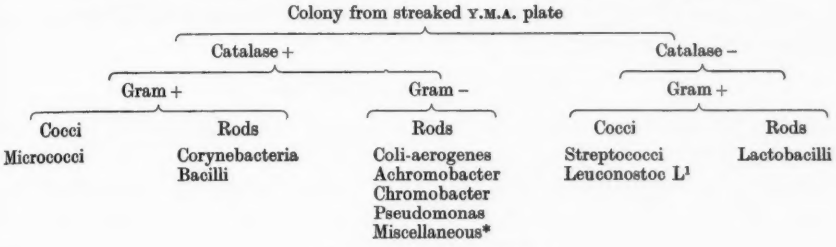
Methods

From each farm thirty-two milk samples were examined before and after ageing. The following method was used to distinguish the main types of micro-organisms.

From Y.M.A. plates incubated for 3 days at 30° C. showing 150–200 colonies, 10 colonies were picked (using the statistical method of Harrison (7)), inoculated into plain litmus milk and incubated at 30° C. for 3–4 days. Y.M.A. plates were streaked from the litmus milk culture and incubated at 30° C. for 24–36 hr. Where necessary a single purification of the culture was made. This was sufficient for a broad grouping of micro-organisms. A Y.M.A.

slope was prepared from each colony, incubated at 30° C. for 24 hr., and kept in the cold store for further reference. The scheme in Table 5 shows the criteria used for the differentiation of the main types.

Table 5. *Methods of differentiating main groups of micro-organisms*



* Gram-negative or doubtful.

Results

During the first 13 weeks only the initial milk flora was studied and 20 colonies were picked from one plate of the milk from each farm. After this the scheme was modified to include the milk flora of milk aged for 24 hr. at 22° C. The number of colonies was limited to ten from each plate, i.e. four sets of ten colonies were picked weekly.

Table 6. *Percentage distribution of main groups of micro-organisms in fresh milk*

	Farm A				Farm B			
	Hypochlorite	NaOH	Hypochlorite	NaOH	NaOH	Steam	NaOH	Steam
Micrococci	90·0	97·2	72·8	75·3	69·1	37·7	78·5	31·8
Streptococci	2·1	0	0	11·1	5·8	48·8	7·6	43·9
Bacilli	3·2	0	1·0	1·2	21·6	3·8	3·2	0
Achromobacter	2·1	1·1	12·2	3·7	0·7	3·3	6·5	17·1
Pseudomonas	0	0	1·0	1·2	0	2·7	1·0	4·8
Corynebacteria	1·3	0	1·0	0	0·7	1·6	0	1·2
Coli-aerogenes	0	0	6·0	0	0	0·5	0	0
Miscellaneous	0	0	1·0	0	0	0·5	0	0
No growth	1·3	1·7	5·0	7·5	2·1	1·1	3·2	1·2

Table 7. *Percentage distribution of main groups of micro-organisms in aged milk*

	Farm A				Farm B			
	Hypochlorite	NaOH	Hypochlorite	NaOH	NaOH	Steam	NaOH	Steam
Micrococci	—	96·8	49·0	70·5	—	41·8	70·5	38·3
Streptococci	—	0	0·9	12·6	—	24·1	0	5·4
Bacilli	—	0	4·1	0	—	6·1	4·2	0
Achromobacter	—	0	19·9	2·4	—	20·8	11·5	31·5
Pseudomonas	—	0	12·7	3·7	—	0	4·2	23·2
Corynebacteria	—	0	0	0	—	0	0	0
Coli-aerogenes	—	0	6·2	4·8	—	7·4	9·4	0
Miscellaneous	—	0	2·0	1·2	—	0	0	0
No growth	—	3·2	5·2	4·8	—	0	0	1·3

Tables 6 and 7 show the distribution of the main groups of micro-organisms during the different periods for the fresh and the aged samples, expressed as a percentage of the total cultures isolated during each period.

In the fresh samples of milk micrococci predominated with both caustic and hypochlorite treatments at farm A. This was also true of the caustic treatment in farm B, but not with the steam treatment. We can offer no explanation for this. The types of micro-organisms were more restricted with the caustic treatment.

With the aged samples the flora isolated during the caustic treatments shows little change with ageing. With both control treatments there is a general small increase in most of the less dominant groups.

SOURCE OF MICRO-ORGANISMS

An attempt was made to determine the main sources of micro-organisms by taking individual milk samples from all cows contributing to the bulk milk, and by taking rinse samples from the clusters and sampling the udder washing water. The milking units were the only articles coming into contact with the milk, apart from the milk cans which were virtually sterile. The caustic and water rinse solutions had been tested consistently throughout the work and were known to contribute little to the final count of the milk.

The udder washing solutions, the chlorine in which was neutralized immediately on sampling (500 ml. sample neutralized with 1·8 g. sodium thiosulphate sterilized in the sampling bottle) never gave counts in excess of 50/ml., and sometimes gave 0/ml.; tests for coli-aerogenes organisms were always negative in 1 ml. The cluster rinse results show that there were insufficient organisms on the cluster to alter the bulk milk count, being on an average 62×10^3/cluster at farm A and 77 and 37×10^3 respectively on two clusters at farm B, equivalent to 1–2 organisms/ml. if distributed in 10 gal. of milk.

Table 8. *Comparison of colony counts from individual cow samples and the corresponding bulk milk*

	Farm A (weekly samples)							
Individual cow samples	970	1,840	630	2,400	3,680	460	1,665	200
	9,080	11,340	11,600	9,500	1,870	680	620	5,000
	540	50	3,500	625	—	—	—	60
	8,010	10,160	13,560	1,600	—	—	—	—
Mean	4,650	5,847	7,322	3,825	2,058	570	1,142	1,420
Bulk milk	1,390	2,930	9,330	5,900	2,860	5,850	11,300	7,400
	Farm B (weekly samples)							
Individual cow samples	420	1,576	570	460	510	2,860	420	1,960
	220	680	1,000	250	780	130	1,230	8,700
	1,890	1,570	750	2,500	1,600	680	620	430
	950	1,860	6,550	2,140	2,090	870	110	40
	17,400	3,160	1,090	570	3,660	1,490	110	1,730
	270	240	2,800	6,000	2,400	1,950	350	730
	232	920	—	—	—	800	190	150
Mean	3,054	1,429	2,126	1,987	1,840	1,254	432	1,962
Bulk milk	2,620	9,930	6,530	1,400	3,890	1,930	1,620	990

The counts from the individual milk samples are given in Table 8, together with the result from the corresponding bulk milk sample. It can be seen in most cases that the arithmetic mean of counts of milk from individual cows corresponds fairly closely to the bulk milk count. Therefore, in the majority of cases the numbers of organisms in the individual milk samples could account for the numbers in the bulk milk. In 25% of the cases, however, the bulk milk counts were considerably higher, no doubt as a result of dirt being drawn into the machine during milking.

On one occasion the predominant types of micro-organisms were determined from individual samples of these cows whose milk gave counts in excess of 1000/ml. The individual cow samples from farm A were predominantly micrococci. Nearly 85% of the flora of all the bulk milk samples from this farm were micrococci. From farm B the types predominant in the individual milk samples were much more diverse. This was in keeping with the types isolated from the bulk milk.

GENERAL DISCUSSION

It is well established that correct use of steam or sodium hypochlorite, following adequate cleaning of dairy utensils, results in milk of a low bacterial count. The bacteriological results obtained during the control periods on each farm were used to ensure that the control treatments were effective. The fact that there is no difference in bacteriological results between the caustic soda and the control treatments indicates that the caustic treatment gave results similar to the best that can be expected under commercial conditions from steam and hypochlorite. If this work had been done on farms where the control treatments were carried out less effectively, and a higher proportion of the bacterial flora had been contributed by the utensils then considerable differences in bacterial flora and quality of the milk would have been noted.

Perhaps the most important conclusion from this work is that the satisfactory bacteriological results of milk samples have been achieved without brushing the milk bearing surfaces, which is necessary in both control treatments.

Generally, differences in prevailing atmospheric temperature alters the bacterial flora of milk. The fact that this was not observed in this work suggests that such changes in bacterial flora, if they occur, may be associated with imperfect cleaning, though it must not be overlooked that mean atmospheric shade temperatures of 67° F. were only rarely experienced and were only once sustained for as long as three days.

At the outset of this work it was expected that the utensils would contribute a significant proportion of the micro-organisms found in the milk. The results show that the majority of micro-organisms in the bulk milk came from the udder. This was borne out by the similarity of the predominant types in individual cow and bulk milk samples. However, as most bacteria from the normal udder have little effect on keeping quality, those bacteria which influence keeping quality must either come from the utensils or in the course of milking. Although the numbers of bacteria from the utensils were small it is possible that these might have had some effect on keeping quality.

As the utensils contribute so few micro-organisms to the milk either from the control or caustic treatments, we are unable to explain whether the change in the majority microflora at farm B was a result of the change of treatment or not. On both farms the microflora of the milk from the caustic treatment was simpler than that from steam or hypochlorite, and on ageing of the milk for 24 hr. at 22° C. it was observed that with steam and hypochlorite treatments the less dominant members of the flora generally increased in numbers. This was not the case with the caustic treatment.

Because of the effectiveness of the cleaning methods used it was necessary to test relatively large quantities of milk so that determinations of coli-aerogenes organisms could be expressed as numbers/100 ml. The results indicate that these organisms are probably never absent from raw milk (apart from small aseptic samples) though it may be necessary

to examine large volumes of milk to find them. As different types of coli-aerogenes organisms under different storage temperatures may multiply at different rates (8), it seems that if the results are used to indicate contamination, they cannot be correctly interpreted unless the tests are done on fresh milk. Since no coli-aerogenes organisms were found in 10 ml. quantities taken from individual cow samples by hand following good udder washing, and no organisms of this type were found on the utensils, then the immediate source of coli-aerogenes organisms found in the bulk milk must have been direct from the immediate surroundings. Such contamination would include organisms from the outside of the udder and dirt picked up from the floor, the milkers' hands and the flanks of the animals during handling of the clusters. The improved udder washing technique used in the latter part of the experiment did not bring about an improvement in coli-aerogenes results of the milk.

The bacteriological tests and some of the flora determinations did not agree. The coli-aerogenes results from MacConkey's broth gave values sometimes as high as 11×10^6/ml. in the aged milk when the total count was only a quarter to a half of this. Parallel determinations of the percentage of these organisms from 30° C. Y.M.A. plates showed that only a small percentage or none of the colonies isolated were coli-aerogenes. This discrepancy indicates that present methods of determining different fractions of the bacterial flora of milk by plating may be most misleading. This may be because of inhibition of some sections of the flora. Thus if it is necessary to make a bacteriological analysis of any material it may be necessary to use selective media for all the different fractions which it is desired to study.

SUMMARY

Two farms, using direct-to-can milking, were used to compare utensil cleaning with hot detergent solution followed by hypochlorite on one farm and steam on the other, with immersion of the complete milking equipment in 5% caustic soda between milkings. Control and caustic treatments were used alternately for periods of 8 weeks for a total of 8 months.

Bacteriological tests made at weekly intervals on fresh milk and milk aged for 24 hr. at 22° C. showed little difference between the control methods, which involved brushing the milk contact surfaces, and the caustic soda method where there is no brushing.

The majority of micro-organisms in the bulk milk came from the udder and the utensils contributed little to the milk. It appeared that where utensils are satisfactorily cleaned, milking technique will be the factor which mainly affects keeping quality.

A study of the predominant flora revealed that milk from equipment cleaned by immersion in caustic soda had a simpler flora (mainly micrococci) than the control treatments.

We should like to thank the owners, management and staff of these two farms, Sir Edward Goschen and Mr Wood of Jesmond Hill Farm, Pangbourne, Berks, and Mr Hatt and Mr Blakey of Combe End Farm, Whitchurch, Oxon, for their helpful co-operation in this investigation.

REFERENCES

(1) GARDNER, E. R. & BERRIDGE, N. J. (1952). *J. Dairy Res.* **19**, 31.
(2) JOHNS, C. K. (1933). *Sci. Agric.* **13**, 460.
(3) MINISTRY OF AGRICULTURE AND FISHERIES (1954). *Advisory Leaflet*, no. 422. London: H.M.S.O.
(4) HOY, W. A. (1951). *N.I.R.D. Annual Report*, p. 51.
(5) HOBBS, B. C. & WILSON, G. S. (1942). *J. Hyg., Camb.*, **42**, 436.
(6) THIEL, C. C., CLOUGH, P. A. & CLEGG, L. F. L. (1955). *J. Dairy Res.* **22**, 156.
(7) HARRISON, J. (1938). *Proc. Soc. agric. Bact.* no. 12.
(8) GARVIE, E. I. & ROWLANDS, A. (1952). *J. Dairy Res.* **19**, 133.

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584. SPORE-FORMING ORGANISMS IN COMMERCIAL STERILIZED MILK

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There have been few recorded descriptions of spoilage of sterilized milk. Leitch (1) described the experiences of a north country milk depot where the treated milk began to curdle a short time after delivery. He isolated a spore-forming bacillus, stated to be similar to *Bacillus mycoides*, from the bottled milk and from the washed bottles, and suggested sterilizing the washed bottles in an autoclave before filling them.

Hiscox & Christian (2) reviewed the troubles commonly met with in sterilized milk treated at 212° F. for 30 min. They described three types of spoilage: (1) Digestion with a neutral or alkaline reaction such as *B. subtilis* and *B. cereus*. (2) Acid coagulation with subsequent contraction and slight digestion of the curd with separation of clear whey (unidentified organism). (3) The production of coconut or carbolic taints in milk showing no visible change (*B. circulans*).

Nichols (3), working with canned evaporated milk, used the classification suggested by Gibson & Topping (4, 5, 6). Of fifty-two cultures isolated from both faulty and apparently normal tins, Nichols found thirty-six strains of *B. subtilis*, eight of *B. licheniformis*, three of *B. pumilus*, two of *B. brevis* and one of *B. cereus*. Probably the most heat resistant were *B. subtilis*, *B. licheniformis* and *B. pumilus* which were found to occur as common contaminants in milk.

Morgan (7) described the typical feathering of sterilized milk in the initial stages of sweet curdling when added to tea or coffee. He found this to be caused by *B. subtilis* and heat-resistant strains of *B. cereus*. He also mentioned a bitter taint caused by *B. thermoacidurans* (*B. coagulans* of Smith, Gordon & Clark (8)), and an oxidized flavour brought about by *B. circulans*.

Clegg (9) described spoilage in sterilized milk caused by spore-forming thermophiles. The milk clotted in the bottle after processing before it left the creamery. This was observed at several creameries where the milk was uncooled and was stacked in solid blocks of crates. Clotting of sterilized milk within a few hours of processing could only have been caused by thermophiles, the only bacteria with the necessary rate of growth. Avoiding keeping the milk hot proved an effective remedy, though occasional complaints are still encountered, because thermophiles are present in most sterilized milk and continue to be a potential source of danger. Further, as the absence of mesophilic spore formers in raw milk is rare, the type of spoilage in milk, where the treatment has been inadequate to kill all spores, will depend on the conditions after treatment.

In the summer of 1952, workers at this Institute carried out an investigation into the batch process of milk sterilization (10). During this work a number of strains of exceptionally heat-resistant spore-forming organisms were isolated from spoiled bottles.

The work recorded in this paper is a study of the cultural and biochemical characteristics of these organisms, with an attempt to identify them with cultures previously described in the literature.

METHODS

Isolation of cultures

Several commonly used laboratory media were compared for isolating spore-forming bacteria from heated milk. Milk-starch agar (M.S.A.) was selected as the most suitable. This consisted of nutrient agar (1% Evans peptone, 1% Lab-Lemco, 0·5% NaCl and 2% agar) with the addition of 1% separated milk and 0·1% soluble starch. The addition of starch was suggested by the work of Murrell, Olsen & Scott(11) who found that it prevented the inhibitory effect on spore germination brought about by certain unsaturated fatty acids present in the medium. Mossel & Drion(12) have also adopted this practice and add 0·1% soluble starch to tryptone-glucose-yeast extract agar.

Cultures were isolated from heated milk by streaking from samples showing spoilage on to plates of milk-starch agar which had first been dried for 24 hr. at 30° C. to prevent the spreading of colonies.

Colonies were picked from these plates, the thermophiles into autoclaved milk (120° C. for 30 min.) and the mesophiles into nutrient broth (1% Evans peptone, 1% Lab-Lemco and 0·5% NaCl). This process of streaking and colony picking was repeated 3 or 4 times to ensure purity of the cultures. They were then stored on milk-starch agar slopes in the cold store at about 4° C.

All the mesophiles showed good growth at 30° C. whether they had been isolated at 24, 30 or 45° C. The thermophiles were originally isolated at 63° C., but it was found that on artificial media they grew better at 55° C. All tests of mesophiles were therefore carried out at 30° C. and of thermophiles at 55° C. (A water saturated atmosphere was maintained for the thermophiles by incubating cultures in a small closed incubator with a trough of water on the bottom shelf.)

Morphology

The polychrome methylene-blue stain described by Anderson, Moehring & Gunderson(13) was found to be useful for spore-forming bacteria, particularly for demonstrating spores in milk films. The method of application was modified for this purpose. The film was air dried, fixed by heat and the stain applied for 1 min. while the slide was held over a flame and heated gently until it steamed. It was then washed carefully by immersion, first in cold tap water and then in distilled water, and air dried.

The Gram stain used was the modification recommended by the Coliform Sub-Committee(14).

While polychrome methylene-blue stained spores more satisfactorily than the Gram stain, it was found after some experience that this method provided no additional information with pure cultures. Therefore only the Gram stain was used in the microscopical examinations for the identification of pure cultures and the polychrome methylene-blue stain used only for studying spores in milk. The cultures were examined after 24 hr. on M.S.A. at 30 or 55° C. Those cultures which showed few or no spores after 24 hr. incubation were re-examined after a further 24 hr.

Colony appearance

Colonies were examined after 48 hr. growth on M.S.A. plates at 30° C. and usually after 24 hr. growth at 55° C. A few strains of thermophiles grew slowly even at this temperature and were examined further after 48 hr.

Cultural and biochemical tests

In testing the range of growth temperature, cultures were inoculated on to M.S.A. slopes and incubated for periods up to 3 weeks in water-baths, the thermophiles at temperatures ranging from 28 to 75° C. and the mesophiles in the range 9–55° C.

Changes in litmus milk were observed in milk that had been steamed on 3 consecutive days and incubated to check for sterility before inoculation.

The following tests were carried out using the methods described by Smith, Gordon & Clark (15); growth on proteose peptone acid agar at pH 5, growth in 4% NaCl (contained in nutrient broth for the mesophiles and in nutrient agar slopes for the thermophiles), hydrolysis of gelatin, starch and casein, and citrate utilization.

The fermentation of glucose, arabinose and xylose also followed the methods of Smith *et al.* (15). For mesophiles the inorganic nitrogen basal medium was used, but this was unsuitable for thermophiles which were cultured on nutrient agar slopes. One ml./l. of a 1·6% alcoholic solution of brom-cresol purple was added as an indicator since this was found to be more satisfactory than the aqueous solution originally recommended.

The medium used for the production of acetylmethylcarbinol (AMC) was that of Smith *et al.* (15) but the method of testing was with the addition of α-naphthol and KOH to the incubated culture fluid (16). One ml. of the culture was placed in an 18 mm. test-tube and 0·5 ml. of a 6% solution of α-naphthol in absolute alcohol and 0·5 ml. of a 16% aqueous solution of KOH added. The mixture was shaken vigorously to aerate it thoroughly and was also warmed slightly. The depth of colour was increased by allowing the tubes to stand on the bench for about 10 min.

The reduction of nitrates to nitrites was tested for by the method described by the Society of American Bacteriologists (17). Three drops of the sulphanilic acid solution and of the α-naphthylamine solution were added to 1 ml. of each broth culture after 1, 3 and 7 days' incubation.

Production of lecithinase. Yolk-agar plates were prepared by mixing equal parts of 5% yolk broth (18), and 4% nutrient agar at 45–50° C. with aseptic precautions and pouring the mixture immediately into sterile Petri dishes. These were incubated overnight at 30° C. to dry and harden the surface of the agar and to check for sterility. Cultures were diluted by mixing one drop in 9 ml. of Ringer's solution and streaking one loopful of this over the surface of the agar to produce well-isolated colonies. The plates were then incubated for 24 hr. at 30 or 55° C. The appearance of an opalescent zone beneath and around the colonies indicated the presence of lecithinase. The production of distinct zones was sometimes aided by standing the plates for a further 24 hr. on the bench after their removal from the incubator.

Anaerobic growth

(i) *Ampoule method.* Nutrient broth plus 1% glucose (glucose broth) in 9 ml. quantities was inoculated with 1 drop from a broth culture of the spore-forming organism. Three ml. of this was immediately boiled under partial vacuum in a soft glass test-tube with a constricted neck. The boiling was continued for 3 min. at atmospheric temperature and for a further 3 min. with the application of gentle heat in a water-bath at 45–50° C. The tubes were sealed while still boiling and incubated, together with duplicate cultures which were kept under aerobic conditions. The ampoules and the aerobic controls were examined for growth after 2 and 9 days' incubation.

As the thermophiles would not grow well in glucose broth, tests for anaerobic growth were made in autoclaved litmus milk.

(ii) *Shake culture method.* The medium used initially in this test was yeast-dextrose agar, but, although this medium supported the growth of mesophilic spore-formers, it was unsuitable for thermophiles and was replaced by milk-starch agar containing 1% glucose. The agar is tubed in approximately 10 ml. quantities and before use is melted in the usual way. Boiling is continued for about 10 min. to remove absorbed oxygen, and the medium is then cooled to 45° C. in a water-bath. Small volumes (*c.* 0·1 ml.) of active broth cultures of the organisms are introduced with sterile 1 ml. pipettes into tubes of medium, and the contents well mixed by sucking up and blowing back, care being taken to avoid bubble-formation. The tubes are cooled immediately in water, and incubated for 2 days at 37° C. for mesophiles or 1 day at 55° C. for thermophiles. Strict aerobes will grow only on the surface and to a depth of a few mm. of the agar, whereas faculative anaerobes will grow on the surface and throughout the entire medium.

These two methods were compared with that of culturing the organisms on agar slopes in the presence of alkaline pyrogallol. Fifty cultures tested by these three methods gave identical results. The shake-tube method has advantages of simplicity and a shorter incubation period.

Anaerobic production of CO_2. The method of Gibson & Abdel-Malek (19) was followed using the medium they recommended for spore-forming organisms; 5% glucose in 4 parts litmus milk and 1 part nutrient agar.

Sporulation in milk. As all thermophiles readily formed spores in milk, this could be observed microscopically using the polychrome methylene-blue stain. Only a few spores were formed in milk by the mesophilic cultures and as these might have been missed by a microscopical examination a cultural method was used. This consisted of inoculating litmus milk, incubating at different temperatures and following the rise or fall in the numbers of spores in the milk during incubation. Aged broth cultures containing an abundance of spores were centrifuged at 2000 r.p.m. for 5 min. to remove clumps and 1 ml. of the supernatant fluid (containing 10^5–10^6 spores/ml.) was inoculated into 25 ml. autoclaved litmus milk in triplicate. This milk was then heated to 85° C. for 10 min. to kill off all vegetative cells, and a portion was plated immediately in milk-starch agar and incubated at 30° C. The tubes containing the inoculated milks were then incubated at 22, 30 and 45° C. and 1 ml. samples were removed at intervals. These were diluted in 9 ml. of Ringer's solution, heated to about 80° C. for 10 min. and the numbers of spores present determined by plating as above. An increase in spore count or an initial decrease followed by an increase was regarded as evidence of sporulation in milk.

RESULTS

The collection of heat-resistant spore-forming organisms previously isolated from sterilized milk was examined by the tests outlined above to determine their identity and to make a general study of their growth characteristics.

Thermophiles

All the cultures of thermophiles appeared similar. When grown at 55° C. they appeared as large Gram-positive rods, with oval, thin-walled spores which were terminal or subterminal and often slightly larger in diameter than the rods, causing the sporangia to

become slightly swollen. On milk-starch agar the colonies were usually small, round, opaque, and white to light grey in colour. Occasionally growth was spreading, forming a thin moist flat film over the surface of the agar. Growth did not appear to be stimulated by the addition of glucose to the medium.

Cultural and biochemical tests. In litmus milk all the cultures produced a firm acid clot in which the litmus was reduced from the bottom upwards. There was no digestion but in some cases the curd contracted sufficiently to expel a little free whey.

The results of the other cultural and biochemical tests are shown in Table 1. Of the thirty-six cultures tested, none grew at 37° C. or below, all grew at 55° C. and, with one exception, at 65° C., but none grew at 75° C.

Positive results were obtained without exception in the following tests carried out at 55° C.: anaerobic growth on milk-starch agar + 1% glucose; growth on nutrient agar; anaerobic growth in litmus milk; acid production in nutrient agar plus glucose.

Negative results were obtained without exception with the following: growth on 4% NaCl in nutrient agar; growth on proteose peptone acid agar; citrate utilization; lecithinase production; acid production from arabinose and xylose; the Voges-Proskauer reaction using proteose peptone glucose broth.

Hydrolysis of starch was usually positive, though three strains showed no change and two were doubtful. Gelatin was not liquefied except for the possible exception of two strains which showed very indefinite zones. Casein hydrolysis was variable, most strains were negative or doubtful though five were strongly positive.

The thermophiles were tested many times for the production of ACM but even with the α-naphthol method (16), which seemed to be the most sensitive of the various modifications recommended, consistently negative results were obtained. As in many respects these cultures resembled *B. coagulans* (8), this negative result was surprising. Since the cultures did not grow well in the proteose peptone glucose broth they were inoculated into sterile separated milk to which 0·5% glucose had been added. In this medium all cultures showed the presence of ACM after 24 hr. at 55° C. when tested by the Barritt method. (A culture of *B. stearothermophilus* Donk was included in both tests as a control and this gave consistently negative results.)

All the thirty-six cultures readily formed spores in litmus milk at 55 and 63° C. some doing so within 6–7 hr.

Mesophiles

All the mesophilic cultures were of a similar type. When grown at 30° C. they showed small Gram positive rods with round to oval thin-walled spores. The sporangia were not swollen and in each culture the position of the spore varied from central to subterminal.

The appearance of the colonies was variable both between strains of the same type and between subcultures of the same strain.

Cultural and biochemical tests. In litmus milk all the cultures produced a soft clot followed by digestion, which always started at the surface of the milk.

The results of the other cultural and biochemical tests are shown in Table 2. All thirty-nine cultures grew well between 15 and 37° C. and all except seven grew at 45° C. There was no growth at 9° C. during an incubation period of 3 weeks, and no growth at 50° C.

At 30° C. all the cultures grew on proteose peptone acid agar at pH 5 and in nutrient broth containing 4% NaCl. They all formed ACM and hydrolysed starch, gelatin and casein. They all failed to produce lecithinase as shown by the egg-yolk test.

Table 1. *Range of growth temperature and characteristics of the thermophilic cultures at 55° C.*

Strain	No. of cultures	Growth on M.S.A. at ° C. 28	37	45	55	65	70	75	Growth on N.A.	N.A. + 4% NaCl	P.P. acid agar	Reactions on Citrate	NO_3 reduction	V.-P.*	Lecithinase	Hydrolysis of Starch	Gelatin	Casein	Fermentation of Glucose	Arabinose	Xylose	Sporulation in milk	Anaerobic growth in M.S.A. + 1% glucose	Litmus milk
A	6	+	−	+	+	+	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
B	1	+	−	+	+	+	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
C	1	+	−	+	+	+	±	−	+	−	−	−	±	−	−	±	−	±	+	−	−	+	+	+
D	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
E	1	+	−	+	+	+	±	−	+	−	−	−	+	−	−	+	−	±	+	−	−	+	+	+
F	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
G	6	+	−	+	+	+	±	−	+	−	−	−	+	−	−	+	−	±	+	−	−	+	+	+
H	3	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
I	7	+	−	+	+	+	±	−	+	−	−	−	±	−	−	±	−	±	+	−	−	+	+	+
J	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
K	1	+	−	+	+	+	±	−	+	−	−	−	±	−	−	±	−	±	+	−	−	+	+	+
L	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
M	1	+	−	+	+	+	±	−	+	−	−	−	±	−	−	±	−	±	+	−	−	+	+	+
N	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
O	1	+	−	+	+	+	±	−	+	−	−	−	±	−	−	±	−	±	+	−	−	+	+	+
P	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+
Q	1	+	−	+	+	+	±	−	+	−	−	−	±	−	−	±	−	±	+	−	−	+	+	+
R	1	+	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+

A dot indicates the same reaction as the first sign above.

M.S.A. = milk-starch agar; N.A. = nutrient agar; P.P. = proteose peptone; V.-P. = Voges-Proskauer.

* This result becomes positive if milk is added to the medium.

Table 2. *Range of growth temperatures and characteristics of mesophilic cultures at* 30° C.

Strain	No. of cultures	Growth on M.S.A. at ° C.						Growth on			Reactions on				Hydrolysis of			Fermentation of				Anaerobic growth in yeast dextrose agar
		9	15	18	22	30	37	45	50	Nutrient agar	N.A. + 4% NaCl	P.P. acid agar	Citrate	NO_3 reduction	V.-P.	Lecithinase	Starch	Gelatin	Casein	Glucose	Arabinose	Xylose	
A	23	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
B	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C	1	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	−	.
D	1	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.	.	+	.
E	3	.	.	.	.	.	.	−	.	.	.	.	.	+	.	.	.	.	.	.	−	−	.
F	2	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.	+	+	.
G	1	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	+	.
H	6	.	.	.	.	.	.	.	.	.	.	.	−	+	.	.	.	.	.	.	.	−	.
I	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.

A dot indicates the same reaction as the first sign above.

M.S.A. = milk-starch agar; N.A. = nutrient agar; P.P. = proteose peptone; V.-P. = Voges-Proskauer.

None of the cultures grew under anaerobic conditions in yeast dextrose agar shake cultures. None of the cultures produced gas in the anaerobic test of Gibson & Abdel-Malek (19).

Using inorganic basal medium all the cultures produced acid from glucose; all except two produced acid from arabinose and all except six from xylose.

Two of the thirty-nine strains failed to reduce nitrates to nitrites and all except two cultures were able to utilize citrate.

Sporulation in milk. There is little published information regarding the sporulation in milk of mesophilic spore-forming organisms. This point is of considerable importance in the heat-treatment of milk and was therefore studied in some detail. The examination of numerous microscopic preparations from each culture gave no conclusive results because the numbers of spores present in the milk were so low that the spores were liable to be overlooked, even with the examination of several fields. Therefore the cultural method described earlier was used. This consisted of heating portions of incubated milk at intervals and determining the numbers of spores. Ten strains were studied at temperatures of 22, 30 and 45° C. All cultures showed an initial fall in the spore count, observable at 12, 24 or 48 hr., indicating considerable germination of the spores. In almost every case this was followed by a gradual increase, though in some cases this was only slight. In no case was there a decline in the numbers of spores to a figure lower than that of the initial drop. These changes were most marked with incubation at 45° C. Although marked increases did not occur in some samples until after the milk had clotted, there were many samples where increases in spore numbers occurred before that time. It seems that mesophilic organisms form spores in milk, but only in limited numbers while the milk is of normal appearance.

Simplified classification of heat-resistant mesophilic spore-formers

At the outset of this survey both thermophiles and mesophiles were isolated and studied, but as thermophiles were not causing spoilage in the dairies from which samples were obtained, although these organisms were present in the heated milk in large numbers, attention was concentrated on the mesophiles.

The bacterial flora of milk heated to a minimum temperature of 100° C. for 30 min. must of necessity be limited, and an attempt was made to construct a simplified routine method for the identification of these aerobic mesophilic spore-forming organisms. A study of the results of other workers in this field (Gibson (4, 5, 6); Smith, Gordon & Clark (8, 15); Knight & Proom (20)) showed that many of the tests gave little relevant information for this purpose. The tests selected as the most useful are based on the findings of the above workers. These are: morphology; Voges-Proskauer test; hydrolysis of gelatin and starch; anaerobic growth in glucose broth.

The egg-yolk reaction of McGaughey & Chu (18) was also included because of the numerous references in published literature (e.g. Leitch (1); Morgan (7); McKenzie (21)) to the occurrence of *B. cereus* or its variety *mycoides* in commercial sterilized milk. In this survey, however, no strains of *B. cereus* or its variety *mycoides* were isolated that would survive the minimum treatment of 100° C. for 30 min. It may be that the cultures referred to were in fact *B. licheniformis*, as this is not readily distinguishable from *B. cereus* or *mycoides* except by the test of Gibson & Abdel-Malek (19) or by the egg-yolk reaction which was only introduced in 1948.

A consideration of the findings of other workers on the cultural and biochemical reactions of those organisms, which exhibited the greatest heat resistance together with our own findings, enabled a simplified scheme of differentiation to be drawn up. This is given in Table 3.

Table 3. *A simple method of identifying aerobic mesophilic heat resistant spore-forming bacteria from sterilized milk*

	Egg-yolk reaction	V.-P. test	Hydrolysis of gelatin	Hydrolysis of starch	Anaerobic growth—glucose broth	
B. subtilis	−	+	+	+	−	Type 1. Sporangia not swollen or only slightly; thin-walled spore
B. licheniformis	−	+	+	+	+	
B. pumilus	−	+	+	−	−	
B. coagulans	−	+	−	+	+	
B. megatherium	−	−	+	+	−	
B. cereus	+	+	+	+	+	
B. circulans	−	−	+*	+	+	Type 2. Sporangia swollen; thick-walled oval spore
B. brevis	±	−	+	−	−	
B. macerans	+	−	+	+	+	

* + Usually positive; ± variable; if positive a very restricted zone.

These six tests were tried on a collection of 305 cultures, forty-two of which were isolated from commercial sterilized milk from dairies in Birmingham, Stockport and London. The remaining 263 cultures were isolated from samples of raw milk laboratory 'sterilized' at 100° C. for 30 min.; 189 of these were from bulk tanker milk coming into London sterilizing depots, fifty were from farm milk coming into a Reading milk depot and twenty-four from farm milk received at a milk depot in Bern, Switzerland.

Table 4. *Results of simplified tests for identification of heat-resistant mesophilic spore-formers*

	Number of strains				
		Laboratory sterilized milk			
	Commercial sterilized milk	Bulk tanker milk	Farm milk Reading	Farm milk Switzerland	Total
Group 1					
B. subtilis	33	14	10	8	68
B. licheniformis	2	139	38	14	190
B. pumilus	0	0	0	2	2
Group 2					
B. circulans	7	3	0	0	10
B. brevis	0	29	2	0	31
*B. macerans**	0	4†	0	0	4
	42	189	50	24	305

* The distinction between *B. macerans* and *B. circulans* can be made by fermentation of glucose.
† These four cultures produced gas from lactose but not glucose.

All the tests in this section of the work were carried out at 37° C., as all the cultures grew well at this temperature and the reactions took place more quickly than at 30° C.

The results of the classification based on these six tests are shown in Table 4. Only six species were isolated, of which *B. subtilis* was predominant in sterilized milk and *B. licheniformis* in raw milk.

DISCUSSION

It must be emphasized that we were only interested in heat-resistant spore-forming organisms in this work, as clearly other types would have no influence on effectively sealed bottles of sterilized milk, therefore the types isolated in this survey must not be regarded as representative of all spore formers in raw milk.

Growth range for thermophiles and mesophiles

The temperature growth range of cultures isolated from sterilized milk indicates which storage temperatures should be avoided for sterilized milk and also indicates useful incubation temperatures for routine tests for the keeping quality of sterilized milk.

Table 2 shows that all the mesophilic cultures were capable of growth at 15° C., although growth at this temperature was slow. This suggests that if stocks of sterilized milk are built up in a dairy there could be no certainty of preventing spoilage if the milk is to be stored longer than 1–2 weeks, unless it was ensured that the milk was stored at a temperature of less than 15° C. (60° F.). However, we have not determined whether spore germination is hindered by a temperature of 15° C., as the present work was done on cultures containing vegetative cells and spores. Clearly more work is needed on this aspect.

Tables 1 and 2 show that the temperature ranges of growth of the thermophiles and mesophiles studied are quite clear-cut. The growth ranges for these two types overlap at 45° C. Approximately 84% of the thermophiles and 82% of the mesophiles grew at this temperature. Although the temperature ranges overlap it is not suggested that a temperature should be chosen for routine testing with the idea of growing both thermophiles and mesophiles at once, as these two types would not readily grow together in milk, because of their different reactions. Further, as both would be growing at the limit of their growth range this would be bound to influence the rate of growth. Therefore both types should be tested for separately as required.

In testing thermophiles, 55° C. has been found to be the most suitable temperature for artificial media, but for growth in milk, clotting was more rapid and complete at 65° C., and the results of earlier work suggest that 63° C. may be a more suitable temperature, as this was the temperature at which all the thermophiles were isolated. Most cultures will cause clotting in 24 hr. but some require 48 hr. and this time is also necessary for original isolates.

For mesophiles the middle of the growth range is 22–30° C., but the most rapid growth has been observed at 37° C. While most of the types studied caused clotting or digestion within 48 hr. from a pure culture inoculation, not all were as rapid as this, and primary isolations appear to require about 7 days.

Sporulation in milk

All thermophiles examined readily formed spores in milk, some within 6–7 hr. at 63° C. This is in keeping with the findings of Burton *et al.* (10), who observed a build-up of thermophiles in a commercial sterilizing dairy in the bottle-filling section towards the end of a day's run. This finding could only be explained by the occurrence of sporulation during the course of the run on plant surfaces and in residual milk retained in the bottle filler.

All the mesophiles examined formed spores in milk, but much more slowly than the

thermophiles, and there was no evidence that any of the cultures would give markedly increased numbers of spores. The significance of this should be considered in conjunction with the findings of Ridgway (22) who found sporulation of pure cultures in litmus milk; he has never observed sporulation in raw milk. He suggests that raw milk with mixed infections constitutes a different set of conditions under which sporulation does not occur, at least as long as the milk has any commercial value.

Cultural and biochemical tests

Gelatin test. The gelatin test is one of the earliest used for differentiating micro-organisms but it has always been somewhat unsatisfactory because of the varying times necessary for liquefaction with different cultures. Some workers have recommended 56 days' incubation before cultures can be regarded as non-liquefiers. With thermophilic organisms which will not grow below 45° C. it is necessary to examine the incubated tubes after chilling to determine whether liquefaction has occurred. All the thermophiles examined grew with difficulty in liquid media (except milk), and no growth could be observed in gelatin stabs incubated at 55° C. The modification of the gelatin test recommended by Frazier (23) has overcome this difficulty. The gelatin is incorporated with nutrient agar in Petri dishes and the culture streaked on the surface. After 48 hr. incubation the surface of the plate is flooded with an acidified mercuric chloride solution to give an opaque appearance to the unhydrolysed gelatin. This method would appear to be suitable for any organism where the gelatin test is required.

The Voges-Proskauer test. There have been controversies about this test (see Taylor (24); Dowson (25)) as to whether the most sensitive method of determining acetoin should be used, or a less sensitive method which affords an easier separation of bacterial types. With coli-aerogenes organisms undoubtedly the latter is desirable because most of these organisms produce at least a trace of acetylmethylcarbinol. With the spore-forming thermophiles tested here a different situation is presented. With an insensitive method all would have been consistently negative. Therefore it was necessary to choose the most sensitive method, i.e. that of Barritt (16). With thermophiles there is a further complication in that the glucose-proteose peptone broth recommended (17), does not readily support the growth of thermophiles. The use of sterile separated milk, to which 0·5% glucose had been added, overcame this difficulty and produced vigorous growth of all thermophiles tested. This enabled a definite positive Voges-Proskauer reaction to be obtained.

Tests for anaerobic growth

The initial method used of culturing in sealed evacuated ampoules containing glucose broth was found to be unreliable as this is not a sure method of removing all oxygen even though the liquid appears to boil. Furthermore, it is tedious. The Burri shake method can be done in less than one-tenth of the time and gave comparable results except in one or two instances where it was necessary to examine the tubes with a low power magnification.

Bacillus calidolactis (*Hussong & Hammer*)

Gordon & Smith (26) were of the opinion that cultures identified by other workers as *B. calidolactis* were either *B. stearothermophilus* or *B. coagulans*. To form this opinion they must have disregarded the temperature ranges for some of these organisms. When studying

spore-formers isolated from sterilized milk, clearly this is of great importance. Nevertheless, all the thirty-six thermophiles we isolated from sterilized milk were neither *B. stearothermophilus* nor *B. coagulans* but resembled *B. calidolactis* as described by Hussong & Hammer (27, 28). We have compared cultures of all three species. *B. coagulans* differs from *B. calidolactis* in temperature of growth and Voges-Proskauer reaction in broth. *B. stearothermophilus* differs from *B. calidolactis* in gelatin hydrolysis and in the swelling of the sporangia during spore formation. If it is valid to distinguish a different species on two characteristics then we are of the opinion that *B. calidolactis* (Hussong & Hammer) should be so recognized.

The bacterial flora of sterilized milk

The results show that only a limited number of types of bacteria survive most sterilization treatments. The mesophilic flora is limited to *B. subtilis*, *B. licheniformis* and *B. circulans*. It is of interest that all the cultures of *B. circulans* isolated from sterilized milk were from tainted samples.

The only thermophiles examined in this work were all from one area, but were without exception *B. calidolactis*. This confirms the earlier findings of Clegg (9).

We have no exact information about the relative numbers of different types since colonies showing a different appearance picked from the same plate were often found to be similar. However, of the resistant spore-forming organisms the overwhelming weight of numbers of *B. subtilis* in sterilized milk and *B. licheniformis* in raw milk leaves no doubt as to the predominating organisms.

SUMMARY

A study has been made of the cultural and biochemical characteristics of heat-resistant thermophilic and mesophilic spore-forming bacteria isolated from raw and sterilized milk. The tests hitherto used in classifying this group of bacteria have been examined and some modifications suggested; as a result a simplified method of distinguishing different resistant spore-forming organisms in milk is proposed. The types of micro-organisms were few. The thermophiles were restricted to *B. calidolactis* and of the mesophiles *B. subtilis* predominated in sterilized milk and *B. licheniformis* in raw milk.

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REFERENCES

(1) LEITCH, R. H. (1924). *Milk Ind.* **5**, 69.
(2) HISCOX, E. R. & CHRISTIAN, M. I. (1931). *J. Dairy Res.* **3**, 106.
(3) NICHOLS, A. A. (1939). *J. Dairy Res.* **10**, 231.
(4) GIBSON, T. & TOPPING, L. E. (1936). *Proc. Soc. agric. Bact.* no. 9.
(5) GIBSON, T. (1937). *Proc. Soc. agric. Bact.* no. 10.
(6) GIBSON, T. & TOPPING, L. E. (1938). *Proc. Soc. agric. Bact.* no. 43.
(7) MORGAN, G. F. V. (1943). *Proc. Soc. agric. Bact.* no. 31.
(8) SMITH, N. R., GORDON, R. E. & CLARK, F. E. (1952). *U.S. Dep. Agric., Agric. Monograph*, no. 16.
(9) CLEGG, L. F. L. (1950). *J. Soc. Dairy Tech.* **3**, 238.

(10) Burton, H., Akam, D. N., Thiel, C. C., Grinsted, E. & Clegg, L. F. L. (1953). *J. Soc. Dairy Tech.* **6**, 98.
(11) Murrell, W. G., Olsen, A. M. & Scott, W. J. (1950). *Aust. J. sci. Res.* B, **3**, no. 2, 234.
(12) Mossell, D. A. A. & Drion, E. F. (1954). *Ned. melk- en Zuiveltijdschr.* **8**, 106.
(13) Anderson, C. W., Moehring, R. & Gunderson, N. O. (1948). *J. Milk Tech.* **11**, 6.
(14) Report (1949). *Proc. Soc. appl. Bact.* **2**, 10.
(15) Smith, N. R., Gordon, R. E. & Clark, F. E. (1946). *Misc. Publ. U.S. Dep. Agric.* no. 559.
(16) Barritt, M. M. (1936). *J. Path. Bact.* **42**, 441.
(17) Society of American Bacteriologists (1934). *Leaflet* 5, 5th ed., p. 16.
(18) McGaughey, C. A. & Chu, H. P. (1948). *J. gen. Microbiol.* **2**, 334.
(19) Gibson, T. & Abdel-Malek (1945). *J. Dairy Res.* **14**, 35.
(20) Knight, B. C. J. G. & Proom, H. (1950). *J. gen. Microbiol.* **4**, 508.
(21) McKenzie, D. A. (1945). *Dairy Ind.* **10**, 334.
(22) Ridgway, J. D. (1954). Personal communication.
(23) Frazier, W. C. (1926). *J. infect. Dis.* **39**, 302.
(24) Taylor, C. B. (1951). *Proc. Soc. appl. Bact.* **14**, 95.
(25) Dowson, W. J. (1949). *Manual of Bacterial Plant Diseases.* London: A. and C. Black.
(26) Gordon, R. E. & Smith, N. R. (1949). *J. Bact.* **58**, 327.
(27) Hussong, R. V. & Hammer, B. W. (1928). *J. Bact.* **15**, 179.
(28) Hussong, R. V. & Hammer, B. W. (1931–2). *Iowa St. Coll. J. Sci.* **6**, 89.

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585. A PHOSPHATASE TEST FOR PASTEURIZATION OF MILK USING DISODIUM PHENOLPHTHALEIN PHOSPHATE AS SUBSTRATE

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The phosphatase test of Kay & Graham adopted as the official method under the Milk (Special Designations) (Scotland) Order 1951 has proved most valuable. It has, however, certain disadvantages, notably its possible vitiation through accidental contamination with phenol. Tests in which this possible source of error cannot arise should prove useful, at least as a check on the official procedure.

Aschaffenburg & Mullen (1) and Stiven (2) have suggested alternative methods of performing the test. The procedure originally described by Stiven was based on the use of the calcium salt of phenolphthalein phosphate as substrate and was not wholly satisfactory in that it was difficult to elaborate a method allowing of a quantitative assessment. The commercial production of the sodium salt of phenolphthalein phosphate has made possible improvements in the Stiven test.

The preliminary work leading to these improvements is dealt with in Part I of this communication and in Part II an account is given of the results obtained on applying the Kay & Graham, the Aschaffenburg & Mullen and the modified Stiven tests to routine samples of heat-treated milk.

PART I. MODIFICATION OF THE STIVEN METHOD

The use of the sodium salt of phenolphthalein phosphate has made possible the elaboration of a simple, reliable and delicate method of performing the Stiven test, the end result of which can be quantitatively assessed.

Important points to note regarding the modified test are:

(*a*) The substrate is easily prepared and is stable.

(*b*) Phenolphthalein, the end product of the reaction, is not likely to be encountered as an adventitious disturbing factor.

(*c*) The test appears to be as delicate as either the Kay & Graham or the Aschaffenburg & Mullen procedures.

(*d*) Colour standards required for 'matching' to make quantitative assessment are easily prepared.

(*e*) The tint of the end product being pink is easily matched by most people.

(*f*) No 'processing' is required, the reaction being completed by rendering the end product alkaline on adding ammonia.

(*g*) By varying the quantities of milk and substrate both a 'long' test (22 hr. incubation at 37° C.) and a 'short' test (2 hr. incubation) can be carried out.

(*h*) The concentration of the substrate salt is not critical. This is important since commercial preparations of sodium phenolphthalein phosphate vary in their content of free

phenolphthalein. By so diluting substrates that they always contain the same amount of the free dye, comparable assessments can be made with substrates prepared from different batches of the salt.

(1) *Preparation of colour standards*

Table 1 gives the concentrations of phenolphthalein employed to prepare the colour standards.

Table 1. *Concentrations of phenolphthalein employed to prepare the colour standards*

(A stock solution (S) containing 0·05 g. phenolphthalein per 100 ml. ethanol (absolute) is prepared and this is further diluted as shown below.)

No. of solution	Volume of S (ml.)	Volume of ethanol added (ml.)	Weight of phenolphthalein in resulting solution (μg./ml.)
1	0·7	99·3	3·5
2	0·8	99·2	4·0
3*	0·9	99·1	4·5
4	1·0	99·0	5·0
5*	1·1	98·9	5·5
6	1·3	98·7	6·5
7*	1·5	98·5	7·5
8	1·7	98·3	8·5
9*	2·0	98·0	10·0
10	2·3	97·7	11·5
11*	2·4	97·6	12·0
12	2·5	97·5	12·5
13*	3·0	97·0	15·0
14*	3·5	96·5	17·5

All fourteen concentrations are useful when 'standardizing' substrates, but only those marked * need be employed in performing tests, the intermediates being obtained by 'bracketing'.

The controls in the 'long' test consist of mixtures containing scalded* milk 1 ml., soluble barbitone solution (11·54 g./l.) 8 ml. and the above solutions of phenolphthalein 1 ml. Two drops of chloroform are added to each and the control tubes are incubated along with the tests after which 1 ml. 10% ammonia† is added and the controls matched with the tests.

In the 'short' test the control mixtures consist of 2·5 ml. milk (heated to 65° C. for 30–45 min.), 1·5 ml. barbitone solution and the above phenolphthalein solutions 1 ml.: after incubation 1 ml. 20% ammonia is added to develop the full colour.

(2) *Standardizing substrates*

Preliminary experiments showed that the concentration of phenolphthalein phosphate was not critical as similar *increments* in tint were obtained with the same sample of milk using substrates containing 0·16, 0·1 and 0·05 g. of the salt per 1000 ml. Substrates used for routine tests can therefore be standardized in terms of their content of free phenolphthalein, thus allowing the same series of controls to be used with different substrate solutions.

* Tubes, not more than three at one time, each containing 10 ml. milk were placed for 2 min. in a water-bath of boiling water. The water-bath had a capacity of 600 ml. and was sufficiently deep to ensure that the column of milk in each tube was below the level of the boiling water.

† Ammonia solution (sp.gr. 0·880) diluted 10 times.

A. *Preparation for the 'long' test*

(1) A solution containing soluble barbitone 11·54 g./1000 ml. freshly distilled water plus 0·12 g. sodium phenolphthalein phosphate. The solution is brought to the boil and cooled. Chloroform is added to preserve it.

(2) A solution of soluble barbitone alone of the same strength, with chloroform added.

Mix in 100 ml. flask.:

(*a*) Scalded milk 3 ml., 27 ml. of the above substrate (1), and 3 ml. 10% ammonia.

(*b*) Scalded milk 3 ml., 27 ml. barbitone solution (2), and 3 ml. 10% ammonia.

Transfer 10 ml. of (*a*) to a suitable tube—those used for the Kay & Graham test are convenient—and match against the colour standards: if the tint matches no. 8 of the colour standards the product is ready for use and is designated '0·1 g. substrate'. It will usually match one of the stronger controls in which case measured quantities of (*b*) are added, dilution and 'matching' being continued till the tint corresponds to control no. 8.

The required quantity of barbitone solution is added to the stock of the substrate (1) to match colour standard no. 8.

The so-called '0·05 g. substrate' is standardized in the same way, but the initial content of salt is 0·06 g./1000 ml. and for use it is matched against no. 5 colour standard.

Daily observation of these substrate solutions, both visually and absorptiometrically, showed that they are stable and of constant sensitivity even when kept at room temperature over a period of 8 weeks.

B. *For the 'short' test*

In the 'short test' the volume of milk in relation to substrate is increased, the controls being prepared accordingly while the content of phenolphthalein phosphate in the substrate solution is doubled *and the mixtures are rendered alkaline by adding* 2·6 *ml.* 10% *ammonia to each* 30 *ml. substrate before incubation.*

It will be noted that scalded milk is not used for the controls in the 'short' test because, owing to the high concentration of milk protein, matching is rendered difficult if scalded milk is used.

'Standardization' of substrate for the short test is performed as for the long test but the mixtures consist of:

(1) Barbitone solution containing 0·24 g. sodium phenolphthalein phosphate—substrate.

(2) Barbitone solution alone—diluent.

The following are mixed in 100 ml. flask:

(*a*) Heated milk (65° C. for 45 min.) 15 ml., plus 15 ml. of substrate (1) and 3 ml. 20% ammonia.

(*b*) Heated milk 15 ml., plus 15 ml. barbitone and 3 ml. 20% ammonia.

Dilute (*a*) with measured amounts of (*b*) until the tint corresponds to no. 8 colour standard and dilute the stock of substrate accordingly, adding chloroform to preserve it.

(*Note:* It is essential if these substrates are to remain stable that they be prepared from freshly distilled water, and it has been the custom in this laboratory to store them in amber bottles if they are exposed to light.)

(3) *Performing the tests*

A. '*Long*' *test*

For testing each sample four tubes should be used—here again the standard Kay & Graham tubes are convenient. These contain:

(1) Milk under test 1 ml. plus '0·1 g. substrate' 9 ml.
(2) Milk under test scalded 1 ml. plus '0·1 g. substrate' 9 ml.
(3) Milk under test 1 ml. plus '0·05 g. substrate' 9 ml.
(4) Milk under test scalded 1 ml. plus '0·05 g. substrate' 9 ml.

Two drops of chloroform are added, and the tubes shaken and incubated for 22 hr. at 37° C. in a water-bath, after which 1 ml. 10% ammonia is added to tests and controls. All are shaken to disperse any deposit that may have formed and matching is carried out. Tube 2 should correspond to no. 8 colour standard and tube 4 to no. 5. The difference between 2 and 1 and between 4 and 3 gives an 'increment' in terms of the controls and on this the interpretation of the test is based.

B. '*Short*' *test*

This has been used as a 'screening' test and only two tubes have been used. These contain:

(1) Milk under test 2·5 ml. plus '0·2 g. substrate' 2·5 ml.
(2) Milk under test heated to 65° C. for 45 min. 2·5 ml. plus '0·2 g. substrate' 2·5 ml.

Owing to the increased volume of milk it is necessary to render the substrate alkaline before use. It was found by preliminary investigation that this could be done by adding 2·6 ml. 10% ammonia to 30 ml. of substrate prior to setting up the tests. After 2 hr. incubation at 37° C. 0·5 ml. of 20% ammonia is added and matching performed to assess the colour increment.

In the long test in the present work the four tubes were matched by two observers, and in the short test two tubes were matched by four observers. Experience showed that there was remarkably little difference in assessment of increments by different observers.

Examination of a large series of laboratory pasteurized milks (62·5 ± 0·1° C.) showed that:

(*a*) Exposure for 30 min. from the time the milk reached 62·5° C. invariably gave an aggregate increment of less than '10' by the 'long' test and '5' by the 'short' test. (It took on an average 3 min. 15 sec. from immersion for the samples to reach 62·5° C. in the method employed.)

(*b*) With a single exception the same was true for samples exposed for 30 min. from immersion.

(*c*) Exposure for 24 min. from immersion gave, with some exceptions, aggregates of over '10' for the 'long' and over '5' for the 'short' test, but in all of these the assessment by the Kay & Graham method was less than 2·1 Lovibond units (L.U.).

(*d*) The phosphatase seemed to be inactivated in a few samples with great rapidity: in one instance aggregates of '10' and '6' were obtained after only 22 min. exposure, this being associated with a Kay & Graham assessment of less than 1·8 L.U.

In view of these findings a series of comparative tests was made of the 'long' Stiven, 'short' Stiven, Aschaffenburg & Mullen and Kay & Graham reactions in which assessment was made *tentatively* as shown in Table 2.

(4) *Comparison of the four methods using laboratory pasteurized samples*

A series of twenty-five tests was made in which the four procedures were compared. Certified milk was pasteurized at 62·5 ± 0·1° C. for 20, 22, 24 and 30 min. and for 33 min. 15 sec. With the products obtained the four tests were carried out.

Table 2. *A comparison of the long and short Stiven tests with the Aschaffenburg & Mullen and Kay & Graham tests*

	'Long' test increment	'Short' test increment	A. &. M. reading	K. & G. (L.U.)
Underpasteurized	>12	>8	14 and >14	>2·3
Slightly underpasteurized*	10 to 12	5 to 7	10 or >10	>2·1 to 2·3
Adequately pasteurized	<10	<5	<10	<2·1 to 2·1

* The above assessments, and those in subsequent tables, both of the Kay & Graham and of the Aschaffenburg & Mullen tests, are more stringent than those advised for routine work, but in view of the comparative nature of the experiments it was decided to adopt them.

In assessing the results the Kay & Graham tests were examined visually by two observers and verified absorptiometrically, the Aschaffenburg & Mullen tests visually using the comparator and the Stiven tests by the method already described. In those instances in which two observers failed to agree in the Kay & Graham and Aschaffenburg & Mullen tests the opinion of a third observer was taken and the assessment was based on the readings of two out of three.

The pH of each sample was determined electrometrically before pasteurization: the highest reading was 7·00 and the lowest 6·40. Table 3 summarizes the results.

Table 3. *A further comparison of the four methods*

	Pasteurization		
	Inadequate	Slightly under	Adequate
	Reject	Doubtful	Accept
Stiven 'long'	61	13	51
Stiven 'short'	39	16	70
Aschaffenburg & Mullen	21	16	88
Kay & Graham (absorptiometer)	26	12	87
Kay & Graham (visual)	21	10	94

There was good concordance between the visually assessed Kay & Graham results and the Aschaffenburg & Mullen reactions: the Stiven procedures as assessed appear to be too stringent.

When the findings were considered in relation to time of exposure to 62·5° C. the results shown in Table 4 were obtained.

From these findings it appears that:

(1) The Stiven 'long' test, assessed as described, is the most delicate of these procedures for distinguishing between milks exposed to 62·5° C. for 24 and 30 min.

(2) The Stiven 'short' test, although less delicate in this regard, was more delicate than the Aschaffenburg & Mullen or the Kay & Graham tests.

(3) The assessment of both the Stiven tests could be regarded as too stringent in comparison with the other methods, and in relation to these tests increments of 12 to 13 in the long and 7 in the short method might be permissible.

(4) There was little difference between the Aschaffenburg & Mullen and the Kay & Graham procedures assessed visually when exposure to 62·5° C. was for 24 min. or longer.

As the findings in the case of samples tested for 24 min. are, in a sense, critical, further details are given in Appendixes A and B in which observations of particular interest are also noted.

Table 4. *A comparison of the four methods made on milks heated to* 62·5° C. *for different times*

Exposure at 62·5° C.* for ...	20 min.			22 min.			24 min.			30 min.			33 min. 15 sec.†		
Method	Reject	Doubtful	Accept	Reject	Doubtful	Accept	Reject	Doubtful	Accept	Reject	Doubtful	Accept	Reject	Doubtful	Accept
Stiven 'long'	24	1	Nil	21	3	1	16	6	3	Nil	2	23	Nil	1	24
Stiven 'short'	20	5	Nil	13	6	6	6	5	14	Nil	Nil	25	Nil	Nil	25
Aschaffenburg & Mullen	13	7	5	7	5	13	1	4	20	Nil	Nil	25	Nil	Nil	25
Kay & Graham (absorptiometer)	19	1	5	7	5	13	Nil	6	19	Nil	Nil	25	Nil	Nil	25
Kay & Graham (visual)	16	4	5	4	4	17	1	2	22	Nil	Nil	25	Nil	Nil	25

* Before the milks were heated at 62·5° C. each 2 ml. sample was preheated for 5 min. at 37° C. Unless this was done the time between immersion and attainment of 62·5° C. varied considerably.

† This column refers to tests of samples exposed for 30 min. after reaching 62·5° C.—the average time required to reach that temperature was 3 min. 15 sec.

PART II. COMPARATIVE TESTS WITH ROUTINE SAMPLES OF PASTEURIZED MILK

These comparative tests consist of two series, one comprising sixty samples in which the long Stiven test was compared with the Kay & Graham test, the other of 200 samples in which all four procedures were compared.

To evaluate precisely the relative merits of such tests by examining routine samples would involve a prolonged investigation because the percentage of 'doubtful', and therefore critical, specimens is few; indeed a rapid evaluation could be made only in a specially equipped laboratory in which treatment both by the holder and H.T.S.T. methods could be used under conditions resembling those obtaining in commercial pasteurization. The results set forth are given, therefore, with a full appreciation that they are not statistically significant, but it is hoped that their presentation may induce others to test the Stiven method so that adequate numbers of samples may be examined.

In performing the tests with routine specimens the same bases were used as in the experiments quoted in Part I of this communication notwithstanding the suspicion that the assessment of the Stiven test was perhaps too stringent.

(*a*) *Initial series of sixty samples*

(i) Accepted by both the 'long' Stiven and the Kay & Graham tests, 54.

(ii) Rejected by both, 4.

(iii) Rejected by the 'long' Stiven test and 'doubtful' by the Kay & Graham test, 2.

In both samples under (iii) the Kay & Graham reading was 2·3 L.U.

The details of the four rejected were:

(*a*) 'Long' Stiven aggregate >30 Kay & Graham >4 L.U.
(*b*) 'Long' Stiven aggregate >30 Kay & Graham >4 L.U.
(*c*) 'Long' Stiven aggregate >30 Kay & Graham >4 L.U.
(*d*) 'Long' Stiven aggregate >30 Kay & Graham 6 L.U.

Those rejected by the 'long' Stiven test and 'doubtful' by the Kay & Graham reaction gave the following:

(*e*) 'Long' Stiven aggregate >14 Kay & Graham 2·3 L.U.
(*f*) 'Long' Stiven aggregate 17 Kay & Graham 2·3 L.U.

(*b*) *Series of* 200 *samples examined by all four methods*

The results obtained in this series were:

(i) Accepted by all four methods, 182.
(ii) Rejected by all four methods, 9.
(iii) Samples in which the four tests failed to give concordant findings, 9.

Although these nine specimens failed to give concordance the variations were not striking. They were as follows:

Test	Number of specimens		
	Rejected	Doubtful	Accepted
'Long' Stiven	1	6	2
'Short' Stiven	1	2	6
Aschaffenburg & Mullen	0	3	6
Kay & Graham	1	3	5

The distribution of these variations was, then, irregular and was probably due either to technical error or to subjective influences in matching. The details of the nine instances of absence of concordance are given in Appendix C.

SUMMARY AND CONCLUSIONS

1. Details have been given of a modified Stiven test for determining the efficiency of pasteurization in milk.

2. A 'long' form and a 'short' form of the test have been described.

3. The 'long' and 'short' tests have been compared with the Aschaffenburg & Mullen and Kay & Graham tests.

4. The work has led to the following conclusions:

(*a*) The 'long' Stiven test seems best to distinguish between exposure for 24 min. and exposure for 30 min. to 62·5° C., though compared with the other three tests this 'long' Stiven test as assessed in the present work is possibly too stringent.

(*b*) There was little difference between the results of the 'short' Stiven test, the Aschaffenburg & Mullen and the Kay & Graham tests when used for the routine examination of specimens.

(*c*) The ease of performance and the stability of the reagents make the Stiven test convenient to use, and it should be helpful to those who have difficulty in matching blues and yellows.

(*d*) It should be of value as a confirmatory procedure.

REFERENCES

(1) ASCHAFFENBURG, R. & MULLEN, J. E. C. (1949). *J. Dairy Res.* **16**, 58.
(2) STIVEN, D. (1947). *J. Dairy Res.* **15**, 57.

(*MS. received for publication* 7 *January* 1955)

APPENDIX A

Details of findings with samples exposed for 24 min. to 62·5° C.

		Stiven 'long' test		Stiven 'short' test		Aschaffenburg & Mullen test		Kay & Graham test assessed absorptiometrically		Kay & Graham test assessed visually	
Date	pH of sample	Increment aggregate	Category	Increment aggregate	Category	Comparator reading	Category	Lovibond units	Category	Lovibond units	Category
20. xi. 53	6·62	19	R	9	R	<10	A	2·3	D	2·3	D
11. xi. 53	6·60	>17	R	9	R	10	D	2·3	D	2·1	A
2. ii. 54	6·54*	16	R	6	D	<10	A	2·2	D	2·1	A
1. xii. 53	6·50	>15	R	11	R	10	D	2·2	D	2·1	A
25. xi. 53	6·49	>15	R	6	D	6	A	2·0	A	1·8	A
16. xi. 53	6·56	>14	R	11	R	10	D	2·0	A	1·8	A
23. xi. 53	6·74	>14	R	>10	R	<10	A	2·3	D	2·1	A
29. i. 54	6·50	14	R	5	D	>6	A	2·0	A	<2·1	A
12. xi. 53	6·62	>13	R	>3	A	6	A	1·4	A	1·5	A
18. xi. 53	6·64	>13	R	4	A	6	A	1·8	A	<1·8	A
26. xi. 53	6·47*	>13	R	3	A	6	A	1·7	A	>1·5	A
9. ii. 54	6·65	>13	R	>4	A	<6	A	1·9	A	1·8	A
5. ii. 54	6·66	13	R	>4	A	<6	A	1·7	A	1·8	A
5. i. 54	6·72	13	R	>3	A	10	D	1·8	A	1·8	A
13. xi. 53	6·56	>12	R	>6	D	>6	A	1·5	A	1·5	A
24. xi. 53	6·70	>12	R	4	A	<6	A	1·8	A	<1·8	A
27. xi. 53	6·53	12	D	6	D	<6	A	1·9	A	1·8	A
8. xii. 53	6·50	11	D	4	A	14	R	2·1	A	>2·1	D
29. xii. 53	6·65	>10	D	>1	A	6	A	2·3	D	>2·3	R
7. i. 54	7·00	>10	D	3	A	>6	A	1·9	A	1·8	A
12. ii. 54	6·72	>10	D	5	D	<6	A	1·5	A	<1·8	A
6. i. 54	6·68	10	D	3	A	<6	A	1·4	A	1·5	A
19. xi. 53	6·54	9	A	1	A	<6	A	1·5	A	1·5	A
15. xii. 53	6·66	>8	A	3	A	<6	A	1·5	A	<1·8	A
26. i. 54	6·40†	6	A	3	A	6	A	1·7	A	<1·8	A

R = underpasteurized, D = slightly underpasteurized, A = adequately pasteurized, these being assessed on the basis described in the text.

* Further details regarding these two samples are given in Appendix B.

† It will be noted that specimen 26. i. 54, accepted by all tests after 24 min., was unusually acid.

APPENDIX B

APPENDIX B

Details of two specimens in which the long Stiven test gave doubtful results after the milks had been heated to 62·5° C. *for* 30 *min.*

Method	Specimen 2. ii. 54, pH = 6·54 Exposure times (min.)										Specimen 26. xi. 53, pH = 6·47 Exposure times (min.)									
	20		22		24		30		33·25		20		22		24		30		33·25	
	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category	Increment or reading	Category
Long Stiven	24	R	19	R	16	R	10	D	6	A	17	R	16	R	>13	R	10	D	10	D
Short Stiven	8	R	7	D	6	D	1	A	Nil	A	7	D	4	A	3	A	1	A	Nil	A
Aschaffenburg & Mullen	18	R	10	D	<10	A	6	A	<6	A	<10	A	6	A	6	A	6	A	<6	A
Kay & Graham (absorptiometer)	2·8	R	2·4	R	2·2	D	1·5	A	1·6	A	1·8	A	1·7	A	1·7	A	1·5	A	1·4	A
Kay & Graham (visual)	>3	R	2·3	D	2·1	A	1·5	A	1·5	A	1·8	A	1·8	A	1·8	A	1·5	A	1·5	A

APPENDIX C

Details of the nine routine samples which did not, in the series of 200 *tests, give concordant results*

'Long' Stiven test		'Short' Stiven test		Aschaffenburg & Mullen test		Kay & Graham test (visual)	
Increment aggregate	Category	Increment aggregate	Category	Comparator reading	Category	Lovibond units	Category
26	R	9	R	10	D	>2·5	R
12	D	3	A	10	D	1·5	A
12	D	1	A	<10	A	2·3	D
12	D	6	D	<6	A	1·8	A
11	D	3	A	6	A	<1·5	A
11	D	7	D	10	D	2·1	A
11	D	3	A	<10	A	1·5	A
4	A	5	A	<10	A	2·3	D
1	A	Nil	A	<6	A	2·3	D

The degree of discordance was not marked except for the number categorized as 'slightly underpasteurized' (doubtful) by the Stiven long test. The variations are otherwise quite fortuitous, indicating that they were due either to subjective or to technical factors.

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586. COLOUR CHANGES IN HEATED AND UNHEATED MILK

IV. THE THEORETICAL BACKGROUND TO THE REFLECTANCE CHANGES IN SEPARATED MILK

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(With 1 Figure)

It has been shown in previous papers of this series (1, 2, 3) that changes of two general types may occur in the reflectance of separated milk. These are:

(*a*) A decrease in the reflectance which is greater at the blue end of the visible spectrum and becomes progressively less towards the red end (1). This change is caused by heat treatment at temperatures above approximately 90° C. and is associated with the 'browning reaction'.

(*b*) An approximately constant change in the reflectance level throughout the visible spectrum. A number of factors can bring about this type of change: one example is the heating of milk to temperatures of 70° C. and above, when the reflectance rises irreversibly throughout the spectrum (2). Change of the temperature at which the measurement is made, or change in the chemical composition of the milk, can cause similar changes in unheated milk (4).

It seems desirable at this stage to consider theoretically the factors which influence the reflectance of milk, in order to obtain some insight into the physical changes in the milk structure which are responsible for the reflectance changes.

THE SCATTERING OF LIGHT IN SEPARATED MILK

Determination of the reflectance of milk consists of a measurement of the amount of incident light scattered backwards by the milk and contained in a certain solid angle determined by the measuring instrument. The theory of the scattering of light by inhomogeneous solutions and suspensions of particles is highly complex. The limiting case of scattering by spherical particles with linear dimensions considerably smaller than the wavelength of the incident light was first considered by Rayleigh (5). The theory was extended to the case of particles having dimensions of the same order as the wavelength of the incident light by Mie (6).

In the form of the Rayleigh equation used by Oster (7) the intensity of the scattered light from a suspension of uniform spherical particles is given by

$$I=\frac{24\pi^3}{\lambda^4}n_0^4\left(\frac{m^2-1}{m^2+2}\right)^2NV^2, \qquad (1)$$

where λ = wavelength of the incident light in air, n_0 = refractive index of the suspending liquid, m = ratio of the refractive indices of the particles and of the suspending liquid, which is real for non-absorbing particles but complex for absorbing particles, N = number of particles per unit volume, V = volume of each spherical particle.

This formula is considered to be applicable where the particle dimensions are less than approximately one-twentieth of the wavelength of the radiation being considered, i.e. less than about 250 Å. in diameter for visible light.

The scattering particles in separated milk are the casein micelles and the soluble proteins, since the small amount of residual fat can be neglected and the remaining components are in true solution. According to Wiegner, quoted by Eilers (8), the soluble protein particles have diameters in the range 50–150 Å. The Rayleigh formula can be applied without reservation to particles of this size.

Studies with the electron microscope by Hostettler & Imhof (9) have shown that the majority of the casein micelles have diameters from 500 to 1000 Å., but that a few particles with diameters up to 8000 Å. may occur.

Most of the casein micelles therefore have dimensions of the order of one-tenth to one-fifth of the wavelength of visible light, while some particles may have dimensions of the order of a whole wavelength. Strictly, within this range of particle sizes, the detailed Mie theory should be applied.

It is possible to show, however, that over the normal range of casein particle sizes no important error is introduced by applying the simpler Rayleigh equation. The full power-series expansion for the scattering equation derived from the complete theory is given by van de Hulst (10). The principal difference between this result and the more restricted case involving small particles is the presence of a multiplying factor

$$\left[1+\frac{6}{5}\frac{m^2-2}{m^2+2}\left(\frac{2\pi r n_0}{\lambda}\right)^2\right], \tag{2}$$

where r is the radius of the particle.

According to Eilers (8), the refractive index of the calcium caseinate-phosphate phase is approximately 1·41, and the refractive index of water is 1·33. If λ is taken to correspond to the middle of the visible spectrum, 6000 Å., then the multiplying factor for particles 1000 Å. in diameter becomes (1–0·16). The deviation from unity corresponds to the correction to be applied to the Rayleigh formula. In view of the uncertainties involved in applying such a formula to a complex medium like milk, and the fact that we are considering only qualitative and not quantitative relationships, an error of this magnitude can be ignored. The Rayleigh equation, Eqn. 1, may then be used to explain the scattering of light from both the casein and the soluble protein particles in milk.

If the constituents of a suspension remain unchanged, the scattering according to Eqn. 1 should be a simple inverse function of the fourth power of the wavelength. This is not in fact so with milk, and its reflectance does not fall continuously with increasing wavelength in the visible spectrum (1). This is presumably due to some components of the milk having complex refractive indices within this range, i.e. absorbing a proportion of the incident energy. For one wavelength, however, the refractive indices of the components should be constant and reproducible, whether real or complex.

For a homogeneous suspension at one wavelength, therefore, the scattered light is given by

$$I=KNV^2, \tag{3}$$

where

$$K=\frac{24\pi^3}{\lambda^4}n_0^4\left(\frac{m^2-1}{m^2+2}\right)^2.$$

For an inhomogeneous suspension containing S types of particle, the total scattered light is

$$I = \sum_{n=1}^{s} K_n (NV^2)_n, \tag{4}$$

where

$$K_n = \frac{24\pi^3}{\lambda^4} n_0^4 \left(\frac{m_n^2 - 1}{m_n^2 + 2}\right)^2.$$

From Eqn. 4 the scattering of light in an inhomogeneous suspension will increase if the refractive index of one or more of the components increases, or if the product (NV^2) for one or more of the components increases. Because of their size and the proportions present, the product (NV^2) is much greater for the calcium caseinate-phosphate micelles than for the soluble proteins, so that the casein micelles are primarily responsible for the colour of raw separated milk. This is in accordance with simple observation.

THE RELATIONSHIP BETWEEN LIGHT SCATTERING AND REFLECTANCE

The Rayleigh equations refer to the scattering of light from an isolated spherical particle, that is, they apply only to suspensions which are so dilute that little of the light falling on any individual particle has been scattered by other particles. This condition does not exist in a highly opaque material such as milk, in which multiple scattering is important.

The variation of the proportion of reflected light with the turbidity of a suspension, the latter quantity being linearly related to the light scattered by each individual particle as given by the Rayleigh result, has been discussed by Pitts (11). The detailed relationship between the proportion of reflected light and the scattering per particle can be computed only for certain assumed values of the parameters. A typical variation of the proportion of reflected light with the turbidity, τ, for values of the parameters typical of a photographic emulsion (in which, however, a small amount of genuine absorption occurs) is given in Fig. 1 (12). Quite large changes in the amount of scattering therefore cause only relatively small changes in the reflectance, particularly at high scattering values. Reflectance can only be taken to be proportional to the amount of light scattered by each individual particle over restricted ranges of the curve.

THE INTERPRETATION OF REFLECTANCE CHANGES

Reversible changes occurring in separated milk in the approximate temperature range 1–50° C.

Experimental data concerning these changes will be presented in a further paper (4). It is sufficient to note here that certain factors, notably pH and temperature of measurement, can cause a rise or fall in the reflectance of separated milk uniformly throughout the visible spectrum. Rise in the pH of milk above pH 6·6 causes a drop in reflectance of about 5 % per pH unit, while a temperature increase of 10° C. in the range 1–50° C. causes a rise in reflectance of about 1 %.

As shown above, the reflectance of milk is primarily determined by the casein micelles. If the reflectance alters within a range of temperatures in which no major chemical changes are occurring, it is highly probable that a variation in the physical state of the casein micelles is responsible. In the absence of chemical change, it is unlikely that the refractive index of the casein will alter, so that the factor K_n in Eqn. 4 will remain constant. The (NV^2) term must therefore be responsible for any changes in reflectance.

It seems probable that changes in the mean size of the micelles are occurring, the scattering varying as the square of the particle volume or as the sixth power of the linear dimensions, with the reflectance varying accordingly. Because of the high power involved, a small change in the dimensions will alter the scattering to a much larger extent.

A change of this type, since it is independent of wavelength and involves no wavelength-dependent change in the refractive index of the scattering particles, will cause a constant proportional increase in the scattering throughout the visible spectrum, and a corresponding increase in reflectance throughout the spectrum. This agrees with the characteristics of the reflectance changes as observed experimentally.

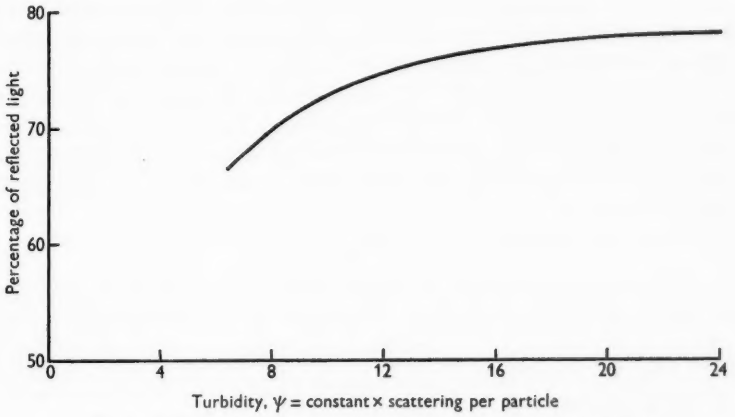

Fig. 1. Computed variation of reflectance with scattered light (Pitts).

The irreversible whitening of separated milk at temperatures of 70° C. *and above*

It has been previously suggested (2) that this rise in reflectance, which may amount to 8% or more, is caused by the denaturation and subsequent aggregation of the soluble proteins. Since the total mass of soluble proteins in the milk must remain constant, aggregation can only occur by a reduction in the total number of particles.

If the density of the soluble protein particles does not change drastically during coagulation, the number of particles, N, per unit volume is approximately inversely proportional to the volume of each individual particle, i.e. is proportional to $1/V$. The product (NV^2) in Eqn. 4 is therefore proportional to V. The amount of light scattered by the soluble protein components of the milk is therefore directly proportional to the mean particle volume, or to the cube of the particle dimensions. It follows that increase in the size of the soluble protein particles will cause an increase in the reflectance of the milk, although it involves a drop in the total number of particles, so that the scattering theory supports the previous explanation for the whitening. The increase in size of the soluble protein particles, although sufficient to affect the reflectance, is not sufficient to allow the aggregated particles to be separated with casein by centrifugation (13).

It has been assumed so far that no change in the refractive index of the scattering soluble protein particles is involved. It is possible that the refractive index may change during denaturation, in which case the reflectance change will be modified from that due

to aggregation alone. A rise in the refractive index will cause a correspondingly increased rise in the reflectance, and conversely a fall will cause a smaller rise. In either of these cases, the change in refractive index cannot be wavelength-dependent within the visible spectrum, or the rise in reflectance would not be uniform throughout the spectrum as is found experimentally.

The browning of milk at temperatures above 90° C.

The changes of reflectance which occur during browning (1), in which the loss of reflectance is much more pronounced at the blue end of the spectrum so that the amount of scattered light may actually increase with wavelength in contradiction to the $(1/\lambda^4)$ law, must be caused by more complex changes in the milk than those considered above. The factor $(m^2-1)/(m^2+2)$ in Eqn. 3, which was a constant function of the wavelength throughout the changes considered previously, is now changing its dependence on wavelength in the visible spectrum. It is probable that this variation is caused by chemical changes which affect the complex refractive index of the suspended particles in ways not yet understood. Change in the size of the particles may not be directly involved, although Ramsdell & Hufnagel (13) have shown that the mean size of the casein particles in milk decreases on autoclaving.

SUMMARY

The theory of the scattering of light in colloidal solutions is considered. As a result it is suggested that the reflectance changes in milk which involve a rise or fall in the general level of reflectance are caused by an increase or decrease in the mean size of the colloidal particles. Whether the particles responsible are the casein micelles or the soluble protein depends on the phenomenon to be explained.

The change in reflectance on the browning of milk, which is dependent on wavelength, is considered to have a more complex cause, involving the production of absorbing molecules.

I wish to express my thanks to Dr E. Pitts of the Research Laboratories of Messrs Kodak Ltd. for helpful discussions on this subject, and also to Dr S. J. Rowland and Dr R. Aschaffenburg of this Institute for their interest and advice.

REFERENCES

(1) Burton, H. (1954). *J. Dairy Res.* **21**, 194.
(2) Burton, H. (1955). *J. Dairy Res.* **22**, 74.
(3) Burton, H. & Rowland, S. J. (1955). *J. Dairy Res.* **22**, 82.
(4) Burton, H. In preparation.
(5) Rayleigh, Lord. *Scientific Papers* (1899), **1**, 92; (1903) **4**, 400. Cambridge University Press.
(6) Mie, G. (1908). *Ann. Physik*, **25**, 377.
(7) Oster, G. (1947). *J. Colloid Sci.* **2**, 291.
(8) Eilers, H. (1947). *Chemical and Physical Investigations on Dairy Products: The Colloid Chemistry of Skim Milk.* Amsterdam: Elsevier.
(9) Hostettler, H. & Imhof, K. (1952). *Ann. agric. Suisse*, N.S., **1**, 307.
(10) van de Hulst, H. C. (1946). *Recherches Astronomiques de l'Observatoire d'Utrecht*, **xi**, part 1, p. 17.
(11) Pitts, E. (1954). *Proc. Phys. Soc.* B, **67**, 105.
(12) Pitts, E. Private communication.
(13) Ramsdell, G. A. & Hufnagel, C. F. (1953). *Proc. XIIIth Int. Dairy Congr.* **3**, 1025.

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587. FLUORESCENCE MICROSCOPY OF FAT IN MILK AND MILK POWDER

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Certain substances, when irradiated by short wave visible light or by ultra-violet light emit a secondary radiation which ceases as soon as the excitation is discontinued. This phenomenon is called fluorescence. It is considered that during irradiation the molecules are transferred from the normal state into an activated state; when they return to the normal state a part of the absorbed energy is emitted as fluorescent light. The time between the absorption and emission is of the order of 10^{-8} sec. Since the energy quantum emitted is smaller than the quantum absorbed, the wavelength of emitted light is longer than that of absorbed light (Stokes's rule). Fluorescence is affected by a number of factors—degree of purity, age and source of the substance, its particle size, its state of dissolution, the nature of the solvent, concentration in solution, the presence of other substances in solution, etc.

A system, either biological (micro-organisms, animal or plant cells and tissues, etc.) or technical, may exhibit fluorescence due to the presence of innate fluorescent substances, e.g. vitamin A, riboflavin, chlorophyll, porphyrins (*primary fluorescence*, *auto-fluorescence*) or it may be rendered fluorescent by treatment with so called 'fluorochromes', materials which are selectively absorbed or adsorbed by certain components of the system (*secondary fluorescence*, *fluorochromy*). Usually the fluorochromes are represented by fluorescent dyes, a number of ordinary biological dyes belonging here, but organic substances of other groups (e.g. alkaloids) may also be involved. Fluorochroming may be regarded as an analogue to the ordinary biological staining process.

In fluorescence microscopy use can be made either of *ultra-violet fluorescence*, the exciting radiation comprising only ultra-violet rays, the wavelengths between 300 and 400 mμ being most appropriate when dealing with biological materials, or of *blue light fluorescence*, when besides ultra-violet rays blue light up to about 500 mμ is employed.

In the present investigation blue light fluorescence was applied. General requirements with this technique are as follows according to Matthaei[6]: (*a*) light source rich in blue emission, with the colour temperature of 3000° K.; (*b*) fluorescence filter transmitting a maximum of energy with a cut-off at 500 mμ; (*c*) Koehler system of illumination, the image of the iris diaphragm of the lamp being projected into the object plane of the microscope; (*d*) screen filter in the eyepiece absorbing the blue light causing the fluorescence, but transmitting the longer wavelengths of the fluorescent light; (*e*) full exploitation of the numerical aperture, both of condenser and objective; (*f*) magnifications kept in reasonable limits to preserve the brightness of image. Of course, only fluorochromes which give fluorescence colours above the wavelength of 500 mμ would be suitable.

EXPERIMENTAL METHOD

A Cooke, Troughton and Simms research type microscope furnished with the outfit for fluorescence microscopy in blue light was used. This outfit employs a high pressure mercury vapour lamp (250 W.) as the light source, the housing of the lamp being connected to the microscope stand by means of a link. The light passes through a lamp condenser, water-cell and deep blue filter which transmits the violet and near ultra-violet rays. The specimen is illuminated in the usual way with a three-lens condenser of numerical aperture 1·40, the iris diaphragm being left fully open. When objectives of × 20 magnification or higher are used, a drop of water, glycerol or paraffin oil is placed upon the condenser to connect it optically with the slide. The slides (about 0·7–0·8 mm. thick) and cover-glasses (of pure white glass) are used. Special attention should be paid to thorough cleansing of them, since even the slightest contamination may cause a disturbing fluorescence. With oil-immersion objective paraffin oil is used as immersion fluid, because of the fluorescence of cedar-wood oil. A yellow filter, complementary to the blue lamp filter, is inserted in the eyepiece. It absorbs blue light quantitatively, but allows green yellow and red to pass through. Usually objectives × 10, × 20 and × 40, and compensating eyepieces × 10 and × 15 were used.

The image in the fluorescence microscope appears very bright and distinct due to the pronounced contrasts, vivid colours and the dark background. However, the light intensity is low, requiring long exposure in photomicrographic work.

RESULTS

(*a*) *Milk*

In the conventional staining technique the fat globules of milk are stained with alcoholic solutions of Sudan III or indophenol, or with aqueous solutions of osmium tetroxide or Nile blue, and the specimens examined in transmitted light.

For the fluorochroming of the fat globules in milk a droplet of milk is mixed on the slide with a droplet of the aqueous solution of fluorochrome, covered with a cover-glass and examined in the fluorescence microscope.

Among a number of fluorochromes reported in the literature to be suitable for the fluorochroming of fats in biological specimens (phosphine, rhodamine B, neutral red, titan yellow, and others) phosphine gave the best results. Phosphine is a brown basic dye consisting of a mixture of nitrates (or chlorides) of chrysanilin, a derivative of acridine, and probably some of its higher homologs. Its aqueous solution (about 0·1%) is brown and does not fluoresce. However, mixed with the fat the latter takes up phosphine and becomes fluorescent.

The fat globules in milk or cream as examined in the blue light fluorescence outfit do not fluoresce, and are scarcely perceivable on a slightly greenish fluorescent background. However, after treatment with phosphine a beautiful, highly contrasted picture of luminous yellowish green fat globules on a black background is obtained. Even the smallest globules, about 0·3–0·5 μ in diameter, are clearly visible. Unlike the image in the ordinary dark ground illumination the globules are not surrounded by diffraction fringes. The fluorescent stain in the globules, sufficient to make them brightly fluorescent, does not reveal itself in the ordinary transmitted light where the fat globules appear untinted.

The brightest picture was obtained with 0·1 % solution of phosphine in distilled water. The fat globules in milk almost instantaneously become fluorescent, which indicates a quick diffusion of the fluorochrome into the globules. Apparently the globule membrane is easily permeable to the molecules of phosphine. The luminosity was somewhat less with the 0·01 % solution of phosphine, while with 0·001 % solution the fat globules generally displayed only a slight fluorescence, the background being slightly greenish.

The colour and intensity of fluorescence were virtually independent of pH between the pH values of 5 and 8.

In milk or cream where the fat has been allowed to crystallize, only the liquid portion of the fat seems to participate in fluorescence, as revealed by switching from fluorescent light to illumination in polarized light. The fat globules with a narrow, non-fluorescent, transparent rim, and a fluorescent core in the fluorescence outfit, turn out to be surrounded by a birefringent peripheral layer when examined in polarized light (preferably with a compensator inserted). The annexes of liquid fat on the globules containing large amounts of crystallized fat show bright uniform fluorescence, while the interior of the globules fluoresces dull-greyish. Spherical aggregates of the needle-shaped fat crystals in large, secondary fat globules fluoresce only slightly, appearing as dark patches. All this demonstrates that the crystalline fat is not able to take up the fluorescent stain, which is true also of the ordinary fat stains (Sudan).

The examination of the fat clumps in the fluorescence microscope distinctly shows that the continuous phase is made up of liquid fat. The fluorescence technique is also a suitable means for studying the behaviour of fat on the milk-air boundary. On the surface of the air bubbles in a milk or cream specimen the spreading of liquid fat from the globules may be observed, the spread fat appearing partly as a belt on the globules, partly as thin patches and layers. Even very thin layers are made visible owing to the strong contrast. Thin specks and patches of fat on the cover-glass, spread there from contact with an occasional fat globule or clump, may also be seen very clearly.

The omission of substances (alcohol, etc.) which may affect the fat-globule membrane and promote a partial coalescence of the globules makes the fluorochroming a suitable procedure for the investigation of the fat distribution in milk, cream, skim milk, buttermilk, etc.

Skim milk as well as *buttermilk* gives a bright picture of yellowish green tiny fat globules on a dark background, whereby even very small globules, about 0·3 μ in diameter, are easily perceivable and measurable. The measuring is highly facilitated by the absence of any diffraction fringes around the globules and the very sharp contours.

In *unsweetened condensed* (*evaporated*) *milk* where the fat is homogenized the luminous tiny fat globules, whose diameters range between 0·3 and about 1 μ, are contrasted against the black background, which greatly facilitates the estimation of the degree of homogenization (e.g. by the method of Farrall, Watts & Hanson modified by Doan & Mykleby (3)). An occasional clustering or clumping of the fat globules is easily revealed.

In *sweetened condensed milk*, where the fat is usually not homogenized, the picture is almost similar to that of ordinary milk.

(*b*) *Milk powder*

The dispersion state of the fat in milk powder particles depends primarily upon the manufacturing technique and the conditions during storage. In roller drying the fat

globules are exposed to rather harsh treatment which damages the globule membrane, with the result that large quantities, up to 95% of the fat in the whole milk powder, are set free. Spray drying affects the emulsion stability to a much lesser extent, and the free fat amounts here only to about 15% of the total fat. In freeze-drying the quantity of liberated fat is between that of the roller-dried and spray-dried powders. When spray-dried whole milk powder during storage is allowed to absorb moisture above a critical level, crystallization of lactose takes place being accompanied by a freeing of the fat (Lampitt & Bushill (4)).

In order to make the fat in powder particles easily discernible under the microscope, and to gain a better insight into the mode of its distribution, biological staining techniques have been employed. Palmer & Dahle (7) stained the powder particles—previously treated with 60% acetone in order to coagulate the proteins partially—with a saturated acetone solution of Sudan III, a small quantity of N/50 HCl being added to increase the intensity of staining. Washburn (8) used differential staining, the fat being stained with Sudan III and the non-fatty matter with haematoxylin. A thin layer of milk powder was embedded in Mayer's glycerol albumin solution spread on a slide, and treated with 65% ethyl alcohol to harden the proteins and prevent their solution. Saturated solution of Sudan III in 70% ethyl alcohol was applied, followed by treatment with 65% alcohol and washing with tap water. Staining with the Harris haematoxylin solution followed. After washing with tap water, the specimen was mounted in Farrant's gum arabic mountant. The fat in roller-dried powders appeared as comparatively large, reddish fragments scattered indiscriminately throughout the blue mass of the particle, many fragments being on the very surface. The fat in spray-dried powders was present in tiny, unfused orange-red globules distributed uniformly in the blue non-fatty portion.

In these staining procedures the treatment of the powder with acetone and alcohol changes the proteins as well as the fat-globule membrane, which in turn may cause changes in the state of the dispersion of the fat in the specimen. It may be expected that by using the fluorochroming technique the proteinaceous components would be affected to much lesser extent, if at all (a parallel to the vital staining of organisms).

Glycerol diluted with distilled water (75 parts concentrated 'Analar' quality glycerol and 25 parts distilled water) and containing 0·1% of phosphine made a suitable mountant, functioning at the same time as the fluorescent stain. Water at this dilution supports the penetration of the fluorochrome into the powder particle, while on the other hand it is not sufficient to disperse the particle. When milk powder (spray-dried whole or skim milk) is mounted in pure glycerol the smaller powder particles do not fluoresce, while the larger ones show a faint greenish primary fluorescence, which, however, fades slowly, starting from the edges of the particle. The fat in powder particles embedded in diluted glycerol (3:1) containing 0·1% phosphine fluoresces brightly yellowish green, while the non-fatty material appears slighly brownish. The specimen has to stand about ½–2 hr. to allow the stain to penetrate through the continuous phase of the particle and into the fat. The background is then only slightly fluorescent, while at the beginning of staining it exhibits pronounced fluorescence.

A beautiful and highly contrasted picture, however, is obtained when use is made of differential fluorochroming with basic fuchsin as counterstain. The fat fluoresces vividly yellowish green, while the non-fatty material is coloured red which, however, does not overlay the yellowish green colour of the fat globules, even very small ones. Examined

in ordinary transmitted light the proteinaceous matter still appears intensely red and shows fine differentiations in the structure of the particles, while the fat is not stained. It is pointed out that acid fuchsin is not suitable as a counterstain in this case, apparently owing to the formation of not easily soluble dye salt with phosphine which is a basic dye, and to pronounced basophily of the casein in powder particle. Only slightly orange coloration of the non-fatty material is obtained with acid fuchsin.

The technique of differential fluorochroming adopted is as follows: a droplet of 0·1% solution of phosphine in diluted (3:1) glycerol and a droplet of 0·1% solution of basic fuchsin in diluted glycerol are mixed on a slide, some of the milk powder to be investigated admixed and the cover-glass placed on. Pressure applied on the cover-glass should be very slight, and any moving of the cover-glass to and fro is to be avoided in order to prevent damaging of the powder particles and alteration in the distribution of fat. By varying the ratio of both solutions the red counter colour may appear more or less deep. In about $\frac{1}{4}$–$\frac{1}{2}$ hr. a very sharply differentiated picture is developed, which is examined by using objective ×20 or ×40 and compensating eyepiece ×10 or ×15.

In a *fresh* or a *well-stored spray-dried whole milk* the tiny yellowish green fat globules appear uniformly distributed in the red pattern of the powder particle. The small particles give the clearest picture. No fusing together of the fat globules, nor any fat patches on the surface of particles which may appear as thin layers on the periphery of particles, can be made out. When the spray-dried whole milk, however, has absorbed moisture during storage with the subsequent crystallization of lactose and liberation of the fat, appreciable amounts of coalesced fat may be seen. It may spread around the air bubbles inside the powder particles as thin greenish layers—even very thin layers being sharply differentiated—and it may appear on the periphery of red powder particles as yellowish green patches or drops. This exuded fat on the surface of particles undoubtedly decreases their wettability. In some cases smaller powder particles are joined together by means of 'fat bridges', which have been formed by the fusing together of large drops of exuded fat still clinging to the particles. Generally, the particles of spray-dried whole milk which contained free fat showed also birefringence in polarized light, due to the crystallization of lactose, while particles in powders with well-dispersed fat failed to show birefringence.

In *roller-dried whole milk*, although some of the fat is dispersed in smaller globules, the bulk of it appears as large globules, fragments and patches in and on the particle. Large yellowish green fat drops may be seen bulging from the deep red proteinaceous frame of the particle. Some of the drops of exuded fat may detach themselves from the particle and rise to the cover-glass where they spread out as thin patches easily perceivable owing to their bright fluorescence.

These highly contrasted pictures of liberated fat effectively demonstrate its easy availability to solvents used in the quantitative estimation of free fat by extraction, as well as its soaking from the roller-dried powders into the packaging material (Lendrich (5); van den Berg (1, 2)).

The particles of *spray-dried skim milk* appear bright red showing only small numbers of minute yellowish green fat globules. Of course, the spherical particles contain a number of air bubbles, more than the particles of spray-dried whole milk (de-foaming action of fat).

By the differential fluorochroming the dispersion of fat is amply made visible also in *powdered ice cream mix*. It is feasible that in a good product the distribution of fat would

be fine and uniform, while coarsely dispersed and partly coalesced fat leaking on to the surface of particles would hinder the incorporation of air into the mix due to anti-foaming action of free fat, or would cause formation of large fat clumps on whipping (buttery ice cream).

It is apparent that differential fluorochroming would be of use also in studying the fat distribution in the particles of cream powder, a product where the fat is extremely liable to liberation.

SUMMARY

The fat globules in milk and cream by taking up the fluorescent dye phosphine from aqueous solution are rendered fluorescent, and show under the blue light fluorescence microscope a bright yellowish green coloration against the black background. Even very small fat globules (in homogenized milk, cream, skim milk, or buttermilk) and very thin fat layers on the milk-air boundary or on glass can be distinguished owing to the high contrast.

By using glycerol containing two fluorochromes, phosphine for the fat and basic fuchsin for the proteinaceous matter, a very sharp differentiation of these two components in milk-powder particles is attained. Differences in the state of dispersion of fat caused by manufacturing technique and storage conditions, and affecting the properties of powders, are clearly made visible.

REFERENCES

(1) Berg, J. C. T. van den (1953). *Molkerei- u. Käserei-Ztg*, **4** (28), 1006.
(2) Berg, J. C. T. van den (1953). *Molkerei- u. Käserei-Ztg*, **4** (29), 1037.
(3) Doan, F. J. & Mykleby, R. W. (1943). *J. Dairy Sci.* **26** (10), 893.
(4) Lampitt, L. H. & Bushill, J. H. (1931). *J. Soc. chem. Ind., Lond.*, **50**, 45T.
(5) Lendrich, K. (1924). *Milchw. Forsch.* **1**, 251.
(6) Matthaei, E. (1952). *Microscopy Conference at University of Melbourne.*
(7) Palmer, L. S. & Dahle, C. D. (1922). *J. Dairy Sci.* **5**, 240.
(8) Washburn, R. M. (1922). *J. Dairy Sci.* **5**, 388.

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588. THE INFLUENCE OF THE CONTENT AND COMPOSITION OF PROTEIN IN MILK ON THE MANUFACTURE OF COTTAGE CHEESE

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An essential in the successful manufacture of cottage cheese is the formation of a coagulum of casein which, when cut into cubes and cooked, will lose moisture at a satisfactory rate. The resultant curd, during the cooking process, should firm up to conform with certain generally accepted and rather rigid standards of body and texture. The maintenance of these standards presents problems to the cheese maker through the influence of a number of factors which may cause day-to-day variations.

In America, where cottage cheese is widely manufactured, much attention has been given to causes of variation, and methods of manufacture to avoid such variations have been suggested.

In New Zealand the manufacture of cottage cheese, on an experimental scale, has been undertaken by the Dairy Research Institute (N.Z.). The American procedure as described by Hales (1), Tretsven (2, 3), Angevine (4), Phillips (5) and Lochry (6), for the manufacture of 'sweet curd' cottage cheese was adopted with certain minor modifications.

Although the results obtained during the early stages were generally satisfactory, some difficulty was experienced on occasions in obtaining a regular rate of acid development. This was eventually overcome by adopting as the starter, a pure culture of lactic streptococcus for which no bacteriophage has yet appeared in the experimental factory of the Institute. From then onwards a high degree of uniformity in the rate of acid development from day to day was obtained.

It was anticipated that no further serious difficulties would be encountered so long as the particular culture remained bacteriophage-free. However, for no accountable reason with regard to the process of manufacture or treatment of the milk, and despite a regularity in acid development which left nothing to be desired, on quite frequent occasions the curd was completely unsatisfactory. The coagulum on these occasions was usually soft and rather flocculent at the cutting stage, although it was further found that even a normal consistency in the curd at cutting provided no guarantee of normal behaviour of the curd during cooking. Moreover, when trouble was encountered, the curd showed a decided tendency to shatter during the cutting and early cooking operations. The main trouble, however, was the almost complete inability of the curd cubes to expel moisture and firm up in a normal manner. Increasing the cooking temperature to a level above normal and extending the duration of the cooking period did not result in an improvement in the condition of the curd, which still failed to dry out and had to be rejected.

The sporadic recurrence of unsatisfactory curd after the essential manufacturing details had been standardized, forms the topic of this investigation.

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With the advancement of the dairying season, a period of hot dry weather prevailed with consequent dry pasture conditions. Preliminary experiments were designed to determine whether the difficulties, previously described, were associated with changes in the composition of the milk, resulting from the abnormal climatic conditions. Subsequently, attention was focused on the influence of milks of different breeds on curd behaviour.

MANUFACTURING PROCEDURE

The milk for cheese-making came from 20 gal. lots drawn from the bulked herd supplies of thirty-five to forty Friesians and eighty to ninety Jerseys. The milk was pasteurized by heating to 160° F. for approximately 12 sec. in a plate heat exchanger and cooling to the setting temperature of 88° F. It was then separated, the skim milk being run into the making vat and 7–8% of starter added. A temperature of 88–90° F. was maintained during the incubation period prior to cutting. At 1 hr. after the addition of the starter, rennet was added to the milk at the rate of 1·5 ml. (N.Z. commercial strength) per 100 gal. milk.

The curd was cut when the pH was between 4·60 and 4·68, as determined by a Beckman pH meter with a glass electrode. Acidity titrations were also carried out on filtered whey. The acidity at cutting, corresponding with the above pH range, was normally between 0·47 and 0·50% lactic acid. The desired acidity and pH was normally attained in approximately $3\frac{3}{4}$ hr. from the time the starter was added.

After cutting with $\frac{1}{2}$ in. curd knives, the curd was allowed to remain undisturbed for 5–10 min. after which time the temperature was raised slowly, either by the admission of steam or hot water to the vat jacket, or by the addition of hot water directly to the vat contents. The rate at which the temperature was raised and the final temperature attained were dependent on the manner in which acid was being produced and on the nature of the curd. Normally a temperature of 120° F. would be reached in 30 min. from cutting.

As soon as the curd was satisfactorily firmed the whey was run off and the curd washed in cold water. At the running stage, control over the nature of the curd practically ceases. No purpose will therefore be served here by proceeding with the discussion on manufacture beyond this stage.

ANALYTICAL METHODS

Solids-not-fat. A 5 ml. aliquot of the sample was weighed into an aluminium dish and dried at 105° C. to constant weight. Day-to-day variations in fat content were found to be negligible, and the mean value of 0·05% was subtracted to give S.N.F.

Total 'protein'. The total nitrogen was determined by a microkjeldahl procedure similar to that developed by Shahani & Sommer (7).

The sample was diluted 1/20 and 5 ml. of the diluate digested with 1·5 g. potassium sulphate, 0·05 g. selenium and 3 ml. N-free sulphuric acid in a 50 ml. microkjeldahl flask until clear. The ammonia from the digest was distilled into boric acid solution, using a Markham apparatus, and titrated directly with 0·025 N hydrochloric acid.

Casein by precipitation. The non-casein nitrogen was determined on 5 ml. of the filtrate obtained after precipitating the casein at pH 4·6 in the manner as described by Shahani & Sommer. This value, when subtracted from the total nitrogen, was taken as the casein nitrogen.

Casein by Walker formol titration. The method described by McDowall & Dolby (8) was used.

Preliminary experiments

Variability in the nature of cheese curd has been attributed to variations in the quality and concentration of certain milk constituents, particularly casein and calcium. The importance of calcium in the rennin coagulum of milk has been recognized by other workers, and difficulties in obtaining a satisfactory coagulum have been overcome, in certain cases, by the addition of calcium chloride to the milk. In America the addition of calcium chloride appears to be common practice in cottage-cheese manufacture.

In this work, analyses of the milk showed no deficiency in calcium. Values ranged from 0·18 to 0·20% as CaO. However, it was considered worthwhile to study the effects of adding calcium chloride to the milk, since, if effective, it could provide an easy means of overcoming the difficulty. The amounts of added calcium chloride and comments on the nature of the curd are given in Table 1.

Table 1. *Effect of adding calcium chloride on the nature of the curd*

Trial	Vat no.	g. $CaCl_2$ per 200 lb. milk	Comment
1	1	17	Curd in vat 1 firmer but both unsatisfactory
	2	Nil	
2	1	30	Both curds satisfactory
	2	Nil	
3	1	45	Both curds satisfactory, vat 1 slightly firmer
	2	Nil	
4	1	45	Both curds satisfactory, vat 1 slightly firmer
	2	Nil	
5	1	45	Both curds unsatisfactory
	2	Nil	
6	1	45	Both curds satisfactory, vat 1 slightly firmer
	2	Nil	

As assessed by these subjective observations, the addition of calcium resulted in no significant improvement in the curd. The slight increase in firmness which resulted from its addition to the milk was not sufficient to correct major defects in body. This result was to be expected since the formation of the coagulum is more dependent on acid precipitation of casein than upon the action of rennet. Calcium, of course, is essentially concerned with rennet action.

Comparison of the behaviour of high-test (Jersey) milk with low-test (Friesian) milk

Early experiments showed that the incidence of unsatisfactory curd was more prevalent when milk of relatively low S.N.F. was used. Yet, the level of S.N.F. in the milk did not provide a reliable criterion of the subsequent behaviour of the curd; milk of relatively low S.N.F. often provided a satisfactory curd, and an unsatisfactory curd was sometimes obtained from milk of relatively high S.N.F. It seemed that a qualitative difference existed between certain milks.

At this stage it was suspected that the source of the milk used might have had some bearing on the nature of the curd. The factory supply was drawn from both the Jersey and Friesian herds on the college farms. For cottage-cheesemaking purposes there was no selection of milk from either breed and, although the supply was predominantly from

Jersey cows, varying amounts of Friesian milk were sometimes inadvertently included with the Jersey milk.

Subsequent experiments were carried out to study the behaviour of curd obtained from Jersey and from Friesian milk manufactured separately. Samples of the milk were analysed for S.N.F., total protein, casein by precipitation at pH 4·6 and casein by the Walker method. The results of four trials are given in Table 2 from which the following will be seen:

(*a*) The curd from Jersey milk was satisfactory in each trial and that from Friesian milk was unsatisfactory.

(*b*) The levels for S.N.F., total protein, precipitable casein and Walker casein were consistently higher in Jersey milk than in Friesian milk.

(*c*) The values for precipitable casein and for Walker casein agreed fairly closely for Jersey milk, but for Friesian milk the Walker casein values were appreciably higher.

Table 2. *Analysis of Jersey and Friesian milk*

Trial	Breed	S.N.F. (%)	Total protein (% N × 6·38)	(C_p) Casein (precipitation pH 4·6) (% N × 6·38)	(C_W) Casein (Walker) (%)	(C_W–C_p)	State of curd*
1	Jersey	9·70	3·60	2·74	2·83	0·09	+
	Friesian	8·86	3·29	2·39	2·63	0·24	−
2	Jersey	9·49	3·83	2·96	3·04	0·08	+
	Friesian	8·56	3·09	2·30	2·72	0·42	−
3	Jersey	9·54	3·80	3·00	2·96	−0·04	+
	Friesian	8·55	3·20	2·25	2·42	0·17	−
4	Jersey	9·47	3·95	3·01	3·20	0·19	+
	Friesian	8·63	3·37	2·46	2·71	0·25	−

* +, satisfactory; −, unsatisfactory.

Effect of adjustment of Jersey and Friesian milks to similar levels of solids-not-fat and casein concentration

Since the Friesian milk was consistently lower in the concentration of S.N.F. and casein it seemed, *a priori*, that the behaviour of the curd might be dependent on these concentrations, and that the peculiar behaviour of the milk of one breed might be produced in the other by adjustment of the concentration.

The Friesian milk was concentrated by the addition of low-heat skim-milk powder. This failed to improve the quality of the curd, even when the concentration of S.N.F. was 1% above that of the Jersey milk.

The Jersey milk was then diluted with water until the casein concentration approximated to that of the Friesian milk. The Walker casein value was used as the index for standardizing since it provided a simple and rapid means of measurement in the factory. The results are given in Table 3, Trials 5–9. Dilution of the Jersey milk in this manner failed to produce an unsatisfactory curd similar to that produced from the Friesian milk.

Since the Walker method gave consistently high values for the concentration of casein in Friesian milk owing to the abnormal distribution of proteins in Friesian milk (discussed later in this paper) a few trials were conducted where the two milks were adjusted to equal precipitable casein concentrations. In every case the Jersey milk produced an unsatisfactory curd similar to that of the Friesian milk. The results of one trial (no. 10 in Table 3)

are given. It will be seen that dilution of the Jersey milk to this extent required 21% of water. It is, of course, generally agreed amongst cheesemakers that dilution of milk to a level of this order presents difficulties in manufacture.

Table 3. *Analysis of Jersey and Friesian milk adjusted to equal casein concentrations*

Trial	Breed	S.N.F. (%)	Total protein (% N × 6·38)	C_p Casein (precipitation pH 4·6) (% N × 6·38)	C_W Casein (Walker) (%)	$C_W - C_p$	Water added (% total)	State of curd
5	Jersey	8·06	3·43	2·67	2·71	0·04	12	+
	Friesian	8·81	2·83	2·05	2·71	0·66		−
6	Jersey	8·14	3·54	2·68	2·64	−0·04	13	+
	Friesian	8·84	3·07	2·23	2·63	0·40		−
7	Jersey	8·49	3·60	2·86	2·90	0·04	11	+
	Friesian	8·65	2·95	2·14	2·70	0·56		−
8	Jersey	8·74	3·55	2·68	2·68	0·00	10	+
	Friesian	8·83	3·44	2·55	2·78	0·23		−
9	Jersey	8·27	3·52	2·70	2·72	0·02	10	+
	Friesian	8·66	3·33	2·53	2·63	0·10		−
10	Jersey	6·95	2·78	2·21	2·26	0·05	21	−
	Friesian	8·64	3·24	2·25	2·61	0·36		−

Effect of mixing Jersey and Friesian milks

Although dilution of Jersey milk to give a concentration of precipitable casein similar to that of Friesian milk produced unsatisfactory curd, it could not be concluded that the peculiar property of Friesian milk could be attributed solely to its relatively low casein concentration. In addition to this quantitative difference it seemed apparent that there was also a distinct qualitative difference between the two milks. It was felt that this concept might be demonstrated by studying the effect of adding Friesian milk to Jersey milk.

In 1925 Koestler (10) showed that milk producing abnormal curd imparted its properties to normal milk, when the two were mixed in the manufacture of certain varieties of cheese. McDowall, Dolby & McDowell (9), on the other hand, found that the influence of normal milk was greater than that of abnormal milk. Although the property studied by these workers is not the same as that presented here, their findings illustrate the controversy which exists with regard to the 'infecting' power of abnormal milk.

The results of a series of experiments, where admixtures of varying proportions of Friesian milk to Jersey milk were used, are given in Table 4. It will be seen that the

Table 4. *Analysis of milk samples from admixtures of varying proportions of Friesian in Jersey milk*

Trial	Friesian milk (%)	S.N.F. (%)	Total protein (% N × 6·38)	C_p Casein (precipitation pH 4·6) (% N × 6·38)	C_W Casein (Walker) %	$C_W - C_p$	State of curd
11	0	9·47	3·95	3·11	3·20	0·09	+
	50	8·76	3·30	2·46	2·86	0·40	−
12	25	9·20	3·75	2·81	3·30	0·49	−
	10	9·50	3·47	2·62	3·23	0·61	−
13	0	9·57	3·97	3·07	3·30	0·23	+
	10	9·46	3·76	2·82	3·20	0·38	−

presence of as little as 10% of Friesian milk was sufficient to render the Jersey milk unsatisfactory.

These results illustrate the power of Friesian milk to impart its peculiar property, with regard to curd formation, to Jersey milk. They also show that the phenomenon cannot be attributed to the low level of precipitable casein since the values in Table 4, where 10% of Friesian milk was included in Jersey milk, compare favourably with those in Table 3, where the Jersey milk was diluted but still produced satisfactory curd.

Distribution of nitrogen in Friesian and Jersey milk

Rowland (11) studied the distribution of nitrogen in the milks of different breeds of cows. His results showed that low values for casein N, expressed as percentage of total N, are associated with pathological disturbances. With milk from normal healthy cows, however, there was no significant difference in the distribution of casein N between breeds.

In Table 5, the results for casein N, expressed as percentages of total N, are given for Jersey and Friesian milks used in these experiments. The values for Jersey range from 76·1 to 79·5 and for Friesian from 70·2 to 76·0. Contrary to the findings of Rowland, these results show a significant difference between breeds ($t = 2{\cdot}269$, significant at $P = 0{\cdot}05$).

Table 5. *Distribution of protein in Jersey and Friesian milks*

	Casein N as percentage total N	
Trial	Jersey	Friesian
1	76·1	72·6
2	77·2	74·3
3	79·0	70·2
4	76·2	73·0
5	77·9	72·4
6	78·0	73·2
7	79·4	72·6
8	79·5	72·5
9	75·5	74·1
10	77·9	76·0
Means	77·67	73·09

The determination of casein by the Walker formol titration, involves titration of all free carboxyl groups after masking the amino groups with formaldehyde. These free carboxyl groups are contributed by all proteins, peptides and amino-acids in the milk. When the titre is multiplied by a constant empirical factor to calculate the percentage of casein, it is assumed that the distribution of casein, relative to the other nitrogenous constituents, is not variable. From the results in this work it is apparent that this assumption is a fallacy and the high Walker casein values for Friesian milk may be explained by the low casein N expressed as percentage total N.

Bannenberg & Hoek (12) have investigated the limitations of the formol titration in calculating the casein and total protein content. They correlated formol titres with total protein and with casein contents, as determined by the Kjeldahl method, over the entire period of lactation. With the advancement of lactation the conversion factor was found to increase. This was attributed to changes in the nitrogen distribution. For mixed herds, however, the influence of stage of lactation did not affect the values significantly and the same conversion factor gave reliable results throughout.

The milk used in the experiments presented here was obtained from Jersey and Friesian cows grazing under the same conditions. Cows of both breeds were in their eighth to tenth month of lactation and were treated similarly in every respect. It seems apparent, therefore, that for the particular cows concerned, there is a breed difference with regard to protein distribution at this rather late stage of lactation.

DISCUSSION

The problem of unsatisfactory curd in the manufacture of cottage cheese at the Dairy Research Institute (N.Z.) can be attributed to the use of milk from Friesian cows. Besides the experimental results submitted it is interesting to record that in the course of an examination of milk supply and cottage-cheesemaking records, made subsequent to this experimental work, it was found that periods in which difficulty was encountered coincided with periods in which Friesian milk was included in the general milk supply of the factory. When, for various reasons, the Friesian milk was diverted for other purposes, no difficulties were encountered.

Inability to produce a satisfactory curd from Friesian milk may apparently result from either of two factors. The first is a low concentration of casein precipitable at pH 4·6. From experiments in which the precipitable casein of Jersey milk was reduced to the same level as that of Friesian milk, it appears that there is a minimal level of casein below which the casein coagulum shows little or no tendency to contract and expel moisture. Excessive dilution of milk used for cottage-cheese manufacture may therefore result in unsatisfactory curd behaviour. The second and more interesting factor is an apparent qualitative peculiarity of the Friesian casein. This was finally demonstrated by adding small quantities of Friesian milk to Jersey milk. The concentration of casein was above the minimal level, yet the curd produced was unsatisfactory. Friesian milk was able to impart its properties to Jersey milk.

There is no evidence to suggest that the peculiar properties of Friesian milk exhibited here are characteristic of the breed. In the U.S.A., where cottage cheese is extensively manufactured, the milk is obtained from Friesian cows and there are no reports of similar difficulties in the manufacturing process. It seems that the peculiarity of the protein may result from some local environmental or nutritional factor which does not exist elsewhere. Further work is required to establish the nature of this factor and discover whether it is associated with a rather dry New Zealand summer, similar to the one during which these experiments were conducted.

SUMMARY

1. The problem of unsatisfactory curd formation in cottage-cheese manufacture has been studied in relation to milk composition.

2. Addition of calcium chloride to the milk did not produce significant improvement.

3. There was a distinct breed difference in the curd formation; Jersey milk produced satisfactory curd whilst Friesian milk invariably produced unsatisfactory curd.

4. Dilution of Jersey milk to the same level of precipitable casein as Friesian milk resulted in unsatisfactory curd.

5. The presence of 10% Friesian milk in Jersey milk rendered the latter unsatisfactory.

6. The Walker formol titration for determination of casein gave significantly high

values with Friesian milk. This is attributed to the different distribution of nitrogen in Friesian milk.

The authors' thanks are due to Mr N. Y. Oetiker and Miss S. E. Quin for technical assistance in the cheese manufacture and laboratory work respectively. They are also grateful to Prof. W. Riddet and Drs C. R. Barnicoat and H. R. Whitehead for their helpful criticism and advice.

REFERENCES

(1) Hales, M. W. (1950). *Sweet Curd Cottage Cheese*, 2nd ed. Chr. Hansen's Laboratory Inc., Milwaukee.
(2) Tretsven, W. I. (1947). *Milk Pl. Mon.* **36** (5), 24–7 and 32.
(3) Tretsven, W. I. (1947). *Milk Pl. Mon.* **36** (6), 26–9 and 32.
(4) Angevine, N. C. (1953). *Milk Pl. Mon.* **42** (2), 32.
(5) Phillips, C. A. (1930 revised 1944). *Circ. Calif. agric. Ext. Serv.* no. 48.
(6) Lochry, H. R. (1931 revised 1948). *Misc. Publ. U.S. Dep. Agric.* no. 119.
(7) Shahani, K. M. & Sommer, H. H. (1951). *J. Dairy Sci.* **34**, 1003.
(8) McDowall, F. H. & Dolby, R. M. (1936). *N.Z. J. Sci. Tech.* A, 17, 619.
(9) McDowall, F. H., Dolby, R. M. & McDowell, A. K. R. (1937). *J. Dairy Res.* **8**, 31.
(10) Koestler, G. (1925). *J. Dairy Sci.* **8**, 28.
(11) Rowland, S. J. (1938). *J. Dairy Res.* **9**, 47.
(12) Bannenberg, H. J. & Hoek, W. van den (1949). *Ned. melk- en Zuiveltijdschr.* **3**, 162.

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589. A CHEMICAL INVESTIGATION OF THE VOLATILE FLAVOUR PRINCIPLE OF CHEDDAR CHEESE

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The problem of the mode of formation and identity of the substance or substances which confer flavour on hard-pressed cheese (in particular Cheddar cheese), has been investigated by many workers during the past fifty years. No one has so far isolated a pure chemical or mixture of chemical substances which could be shown to constitute the flavour material of cheese. Pette (1) recently reviewed the subject with special reference to the hard rennet cheeses. He concluded that more work needed to be carried out in an endeavour to ascertain whether the amino-acids or polypeptides, formed during protein degradation in the ripening cheese, played any part as flavour substances. He further considered that the higher volatile fatty acids played an important part in the development of flavour in many kinds of cheese. Recent work by the present author (2) did not encourage the hope that the answer to the cheese flavour problem would be found by investigation of the amino-acids present in ripening Cheddar cheese. Neither single amino-acids, nor amines, nor mixtures of them as found in mature Cheddar cheese, gave any indication of possessing an aroma or taste resembling that of the typical cheese.

In default of a better approach it was decided to attempt an attack on the problem from the purely chemical angle, i.e. to endeavour to concentrate the flavour substances from cheese and to get some idea of their chemical nature. Suzuki, Hastings & Hart (3), as long ago as 1910, reported that a neutral 'flavour solution' possessing an aroma very similar to that of cheese could be prepared from American Cheddar cheese by steam-distillation methods. Chemical examination of the solution indicated that it contained alcohols and esters, the latter being made up largely of ethanol and acetic acid components. They could not, however, isolate any substance or substances definitely responsible for the characteristic cheese flavour. No one has, since then, attempted to define the chemical nature of the cheese flavour substance. The present paper describes the results obtained in an examination of New Zealand Cheddar cheese along the same general lines as those adopted by Suzuki *et al.* (3).

PREPARATION OF THE CHEESE FLAVOUR CONCENTRATE

Cheeses

All the cheeses used were of 80 lb. weight. Those which were to be examined at intervals during the ripening period were manufactured at the Institute's experimental factory. From time to time, mature cheeses aged 8 months or more were obtained from commercial cheese factories for examination, and in particular for the isolation of the flavour concentrate. Only cheeses which were judged to be of normal or typical flavour were used in the experiments. When a cheese was cut and a portion taken for examination, the remaining pieces were then rewaxed, kept in the cheese cool store and used as required.

Isolation of the volatile, neutral flavour concentrate

Freshly cut cheese was steam distilled in batches of 15–17 lb. Each batch was first treated in small lots with distilled water in a Waring Blendor for 2–3 min. and the whole batch in emulsified form was made up to a volume of approximately 10 l. Dry steam was blown through the cheese emulsion contained in a tinned-steel vessel and the distillate was condensed and collected in a receiver cooled in an ice-salt mixture. Distillation was continued until almost all the cheese flavour appeared to be concentrated in the distillate, the residue possessing only a 'fatty' aroma. Six litres of distillate were normally collected from each batch of cheese, of which the first litre contained the greater part of the flavouring substances. Table 1 shows how the flavour intensity and the pH varied in the progressive fractions of distillate collected.

Table 1. *Details of the fractions collected during steam distillation*

Fraction no. ...	1	2	3	4	5	6	7
Volume distillate (l.)	0·5	0·5	1	1	1	1	1
pH of distillate*	4·18	4·20	4·29	4·36	4·41	4·47	4·58
Flavour assessment	3+	3+	2+	+	+	+	±

* pH of initial cheese 5·20.

The steam-distillable lower fatty acids in the flavour distillates were converted to their sodium salts by adjustment of the reaction to pH 7·1–7·2 by gradual addition of 10% sodium carbonate solution. The distillate or combined distillate was then extracted with one-fifth of its volume of peroxide-free ether. The ether extract was separated and concentrated to a volume of 15–25 ml. over a warm water-bath. The remaining solvent evaporated on standing overnight at room temperature leaving 10–12 mg. of a 'flavour concentrate' which appeared to contain all the 'cheesy' flavouring substances present in the original 15–17 lb. of cheese, assuming that none had been destroyed during the process of isolation.

EXPERIMENTAL

Properties of the volatile, neutral flavour concentrate

The flavour concentrate prepared as described above was a viscous, gummy liquid which solidified to a dull-yellow fatty solid when stored in the refrigerator at 4° C. It had a strong 'cheesy' odour, and when added to the residue of the cheese in the steam-distillation vessel, it conferred on the flavourless material a smell and taste resembling that of the original cheese. There was thus good evidence that the steam-distillation process had not resulted in the destruction through chemical change of the flavour substances to any major extent. The concentrate was subjected to the following examinations:

(i) *Effect of heat.* When a small portion of the material on a stainless steel spatula was gently heated over a bunsen flame, there was first a smell resembling that of Cheddar cheese. This was followed by the emission of a pleasant 'estery' odour and then finally, on stronger heating, by a strong and very characteristic 'rubbery' smell. This rubber-like smell had been noticed in other experiments when cheese was strongly heated during the estimation of volatile fatty acids by the method of Kosikowsky & Dahlberg (4).

(ii) *Effect of oxidation and reduction.* The flavour substances in the concentrate appeared to be fairly stable in weakly acid or neutral solution but to be partially or completely

destroyed by alkali. Warming the concentrate with 2N-NaOH produced a soap-like odour (see (vi), Saponification); the concentrate also lost its 'cheesy' flavour under the influence of oxidizing agents such as $KMnO_4$ and reducing agents such as nascent hydrogen (cf. cardboard flavour (5)). There was also evidence of the presence of unsaturated compounds in the flavour concentrate.

(iii) *Elementary analysis.* Tests for the presence of the elements N, S, P and the halogens were carried out by the methods described by Feigl (6) and Vogel (7) with negative results. Thus it appeared that the concentrate contained only substances composed of carbon, hydrogen and probably oxygen.

(iv) *Solubility tests.* The concentrate was insoluble or only very sparingly soluble in water. It was completely soluble in ether, chloroform, low-boiling petroleum ether and acetone. Gradual addition of excess absolute alcohol to an ether solution of the concentrate precipitated a whitish amorphous compound (consisting about 20% by weight of the concentrate) with wax-like properties. The cheese flavour still remained in the filtrate after removal of this precipitate.

(v) *Specific and group tests.* The flavour concentrate was tested—see Feigl (6) and Vogel (7)—for the presence of the following functional organic groups:

Alcohols, phenols, enols, carbonyl compounds (C=O group), *o*-dioxo- and oxomethylene compounds (—CO—CO— and $—CH_2—CO—$ groups), aldehydes (—CHO group), α:β-unsaturated and aromatic aldehydes (C=C—CHO group), methyl ketones ($CH_3—CO—$ group) (see also Berridge, Zielinska & Barrett (8)), thioketones and mercaptans, carboxylic acids and their derivatives.

The concentrates from some batches of cheese gave a positive dimedone test for aldehydes, and butyraldehyde was isolated and identified. Traces of ethanol were detected in some fractions but no conclusive evidence was obtained for the presence of higher alcohols. Otherwise the group tests gave negative results.

(vi) *Saponification.* After saponification and acidification, the concentrate was subjected to paper chromatographic analysis. Acetic and butyric acids were detected. Ethanol was also shown to be present in the saponified concentrate, its presence being confirmed by demonstration of its conversion to acetic acid on oxidation. The indications were that ethyl acetate and ethyl butyrate, the former ester predominating, were the only two simple esters present in the neutral flavour concentrates.

(vii) *Fluorescence.* Ultra-violet absorption analysis (Beckman Model DU Quartz Spectrophotometer) of the neutral flavour concentrate dissolved in ether or chloroform, indicated the presence of a substance (fluorescing in the ultra-violet with a strong bluish colour) with strong absorption at 2725 Å. This property may of course be quite unconnected with the flavour substances.

Flavour tests using various synthetic substances

In the light of the results of the examination of the flavour concentrate, many substances were tested for possible connexion with Cheddar-cheese flavour. They were examined in their pure state, in aqueous and saline solution at various concentrations, and they were further added singly and in admixture to fresh salted cheese curd and to the cheese residue which remained after steam distillation was completed. In reference to concentration of the flavour substances, it has to be remembered that since the total yield of concentrate containing the flavouring material was not more than 12 mg. from

15 lb. of cheese, which represents one part in about 500,000, then the flavour substances themselves were unlikely to be present even to the extent of one part per million.

In these trials the compounds ethanol, butyraldehyde, ethyl acetate and ethyl butyrate which had been detected in the flavour concentrates, were first tested. There was no indication that they contributed to cheese flavour. Neither did diacetyl nor acetyl methyl carbinol, which had both been shown to be present in young cheese but not in older cheese and the flavour concentrates, contribute in any way. The following substances, which might conceivably have been present in traces in the concentrates, were all tested as described above with negative results:

Alcohols: all normal, *iso-* and tertiary alcohols up to and including *iso*-amyl alcohol.

Aldehydes: all normal and *iso*-aldehydes up to and including *iso*-valeraldehyde.

Ketones: all symmetrical ketones up to and including di-*n*-propyl ketone. All normal methyl ketones up to and including methyl-*n*-amyl ketone.

Esters: all simple esters of ethanol with formic, acetic, propionic, butyric, caproic, pyruvic, succinic and lactic acids.

Others: methyl glyoxal (pyruvic aldehyde), 2:3-butylene glycol.

DISCUSSION

The only compounds identified in the steam distillate from cheese, which contained the flavour principle, were ethanol, butyraldehyde, ethyl acetate and ethyl butyrate; the volatile fatty acid fraction of the distillate was shown by gas-liquid chromatographic analysis to consist of acetic acid together with lesser amounts of both butyric and caproic acids. None of these substances, either singly or in combination, have the odour or taste of Cheddar cheese, so presumably some other compound (or compounds, as is to be understood throughout this discussion) present in trace amount, was responsible for the 'cheesy' flavour. Some of the properties of the flavour principle were however determined, viz.:

(i) It is very volatile.
(ii) It is insoluble in water but easily soluble in ether, chloroform, petroleum-ether and acetone.
(iii) It is probably an organic compound containing only the elements C, H and O.
(iv) It is stable under neutral and acid conditions but is unstable in alkaline solution.
(v) It is decomposed by oxidizing and reducing agents.

Consideration of these properties may be a help in further attempts to identify the flavour substance.

In the flavour concentrate there occurred organic substances which possessed a degree of unsaturation and also substances which had a characteristic ultra-violet absorption spectrum. There was, however, no positive evidence to connect these properties with the flavour substance.

The results of the present investigation are not in agreement with observations of other workers (e.g. Berridge, Hiscox & Zielinska (9), Silverman & Kosikowsky (10)) indicating that the volatile fatty acids are important flavour constituents of Cheddar cheese. The fact that an ether extract of the *neutralized* flavour distillates, prepared in the present work, still retained the typical 'cheesy' flavour, affords strong evidence that the volatile acids are not a source of cheese flavour.

Calbert & Price (11) considered from their results that small quantities of diacetyl (less than 0·05 mg./100 g. of cheese) contribute to typical Cheddar flavour. In some of the present experiments, diacetyl was estimated in flavour distillates of cheese as it matured, using the method of Eggleton, Elsden & Gough (12). The results indicated that diacetyl was present in slowly decreasing amounts up to 3 months from the time of manufacture, but that it was absent in mature cheese in which Cheddar flavour was strongest. There was thus no evidence tending to confirm the conclusions reached by Calbert & Price (11).

It appears, therefore, that the essential constituent responsible for 'cheesy' flavour has not yet been identified by anyone. The problem is at exactly the same stage as that of butter flavour, at the time when there was much experimentation on the formation of volatile acids by butter starter cultures, but an ignorance of the identity of the essential flavouring substance in ripened cream butter, namely diacetyl.

SUMMARY

1. Steam distillates containing the flavouring substance(s) from Cheddar cheese were examined chemically. After the volatile fatty acids had been removed, the only substances identified in the flavour concentrates were ethanol, butyraldehyde, ethyl acetate and ethyl butyrate. None of these compounds appeared to be connected with the cheese flavour.

2. The essential flavour compound(s) could not be identified but some of its properties were elucidated.

3. The findings of other workers that the lower volatile fatty acids, diacetyl and acetyl methyl carbinol contribute to Cheddar-cheese flavour could not be confirmed. Flavour tests with numerous other compounds failed to show that they contributed to the typical flavour of Cheddar cheese.

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REFERENCES

(1) PETTE, J. W. (1953). *Rep. Proc.* 13*th Dairy Congr., The Hague*, **2**, 557–64.
(2) DACRE, J. C. (1953). *J. Sci. Fd Agric.* **4**, 604–8.
(3) SUZUKI, S. K., HASTINGS, E. G. & HART, E. B. (1910). *Res. Bull. Wis. agric. Exp. Sta.* no. 11.
(4) KOSIKOWSKY, F. V. & DAHLBERG, A. C. (1946). *J. Dairy Sci.* **29**, 861–71.
(5) PONT, E. G. (1952). *J. Dairy Res.* **19**, 316–27.
(6) FEIGL, F. (1947). *Qualitative Analysis by Spot Tests*, 3rd ed., pp. 309–424. New York: Elsevier Publishing Co. Inc.
(7) VOGEL, A. I. (1948). *A Text-book of Practical Organic Chemistry including Qualitative Organic Analysis*, 1st ed. (with corr.), pp. 916–35. London: Longmans, Green and Co.
(8) BERRIDGE, N. J., ZIELINSKA, M. & BARRETT, J. (1953). *J. Sci. Fd Agric.* **4**, 472–7.
(9) BERRIDGE, N. J., HISCOX, E. R. & ZIELINSKA, M. (1953). *Rep. Proc.* 13*th Dairy Congr., The Hague*, **2**, 587–91.
(10) SILVERMAN, G. J. & KOSIKOWSKY, F. V. (1953). *J. Dairy Sci.* **36**, 574.
(11) CALBERT, H. E. & PRICE, W. V. (1949). *J. Dairy Sci.* **32**, 515–20; 521–6.
(12) EGGLETON, P., ELSDEN, S. R. & GOUGH, N. (1943). *Biochem. J.* **37**, 526–9.

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590. QUANTITATIVE ESTIMATION OF THE AMINO-ACIDS IN CHEDDAR CHEESE AND THEIR IMPORTANCE IN FLAVOUR

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(With 7 Figures)

Although the development of flavour in Cheddar cheese is very probably a complex process involving the enzymic activity of milk, rennet and bacteria, there seems little doubt that the micro-organisms developing in cheese make a major contribution. Thus, unless the milk is of very poor hygienic quality, the cheese made from raw milk is superior in flavour to pasteurized milk cheese (1). It has also been shown that the flavour can be modified by inoculation of the cheese-milk with lactobacilli (2) or micrococci (3).

A number of investigators have considered the possibility that free amino-acids, which mainly arise from microbial activity, contribute to the flavour of cheese. Their observations have been reviewed by Dacre (4). Experiments involving the addition of amino-acids to fresh curd followed by tasting tests after ripening were inconclusive (5), and suffered from the disadvantage that the additions might result in changes of microflora. Other workers have tasted amino-acids singly or in mixtures, and some conflicting results have been reported (6, 7). It would seem important, before assessing the contribution of amino-acids to cheese flavour, to have accurate information as to their quantity. There are, however, few quantitative data on the free amino-acids of cheese. Harper & Swanson (8) gave figures for nine 'apparent free' amino-acids based on microbiological assay, and Kosikowsky & Dahlberg (9) and Dacre (4) obtained semi-quantitative data by paper chromatography, using comparison with standard spots as a means of estimation. The latter author's results were employed as a basis for tasting mixtures of amino-acids. In contrast to previously reported work (6, 7), when added to fresh curd they conferred no flavour.

It was, therefore, considered that quantitative estimations of the amino-acids in Cheddar cheese would be of interest. The technique used might also provide information about other N-compounds in cheese.

MATERIALS AND METHODS

Cheeses

These were made from raw milk by the normal Cheddar process in the Institute's Experimental Dairy and ripened at 55° F.

Preparation of sample

Four borings were taken at random from the cheese and combined to make about 100 g. The bore-holes were immediately plugged with sterile candles and sealed. The combined sample was mixed with approximately 200 g. fine acid-washed sand and the mixture packed into a stainless-steel perforated cylinder lined with cheese-cloth. Pressure was

then gradually applied to a maximum of 1 ton sq.in.$^{-1}$ using a hydraulic press (10). The expressed liquid, consisting of fat and an aqueous solution containing a little suspended matter, was centrifuged (2000 rev./min.$^{-1}$) and the aqueous layer transferred to a glass Seitz filter. Filtration resulted in a liquid of a straw to brown colour, depending on the cheese; it was transparent but frequently the presence of tiny oil-globules could be detected under the microscope.

Amino-acid analyses

It was felt that no paper chromatographic method was available at the time that would give satisfactory quantitative results and consequently the more reliable but more laborious method of Moore & Stein (11), employing a column of Dowex 50 resin in the sodium form, was adopted. It had the advantage that a high salt concentration could be tolerated so that no prior de-salting of the cheese serum was required. Also, information about peptides in the serum might be obtained by this method. In general, 1 or 2 ml. of a 1/25 dilution of the Seitz-filtered serum were analysed on the column.

RESULTS

Figs. 1–5 show the progressive development of amino-acids in the serum during the ripening of a 40 lb. Cheddar cheese. The results of analysing sera from other ripe Cheddar cheese have been included for comparison (Figs. 6, 7). In Table 1 the quantities of

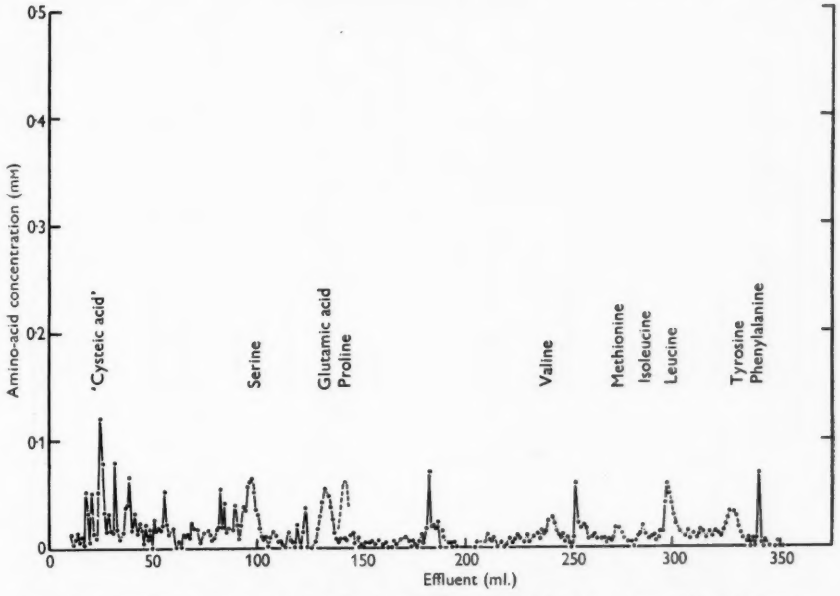

Fig. 1. Separation of amino-acids and peptides in the serum of Cheddar cheese (S1) on Dowex 50 resin. 2-month-old cheese.

individual amino-acids in the sera are presented; they were estimated by summing the data from which the respective peaks have been constructed. Only in the later period of

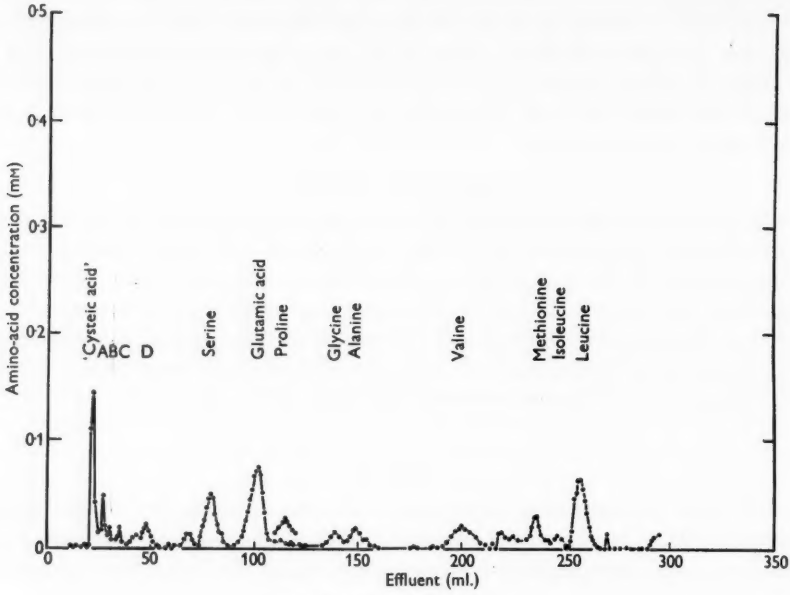

Fig. 2. 3½-month-old cheese.

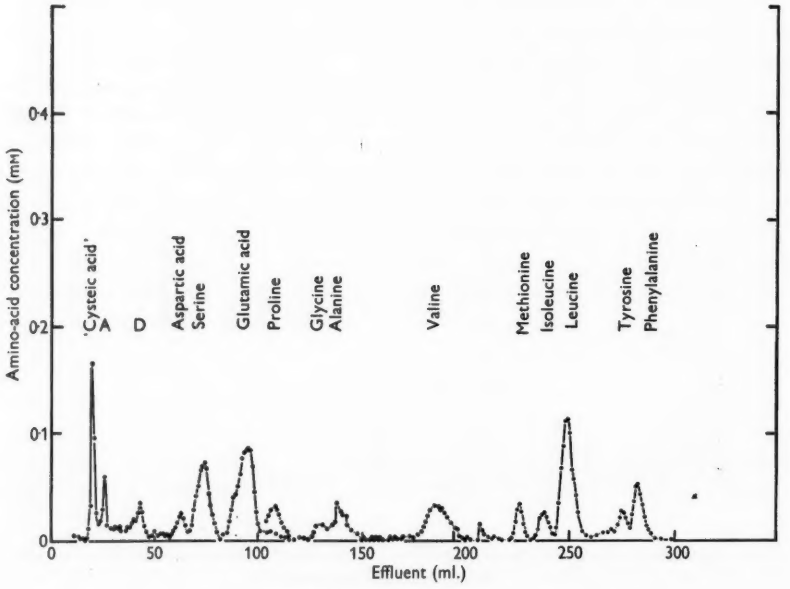

Fig. 3. 4-month-old cheese.

Figs. 2 and 3. Separation of amino-acids and peptides in the serum of Cheddar cheese (S 1) on Dower 50 resin.

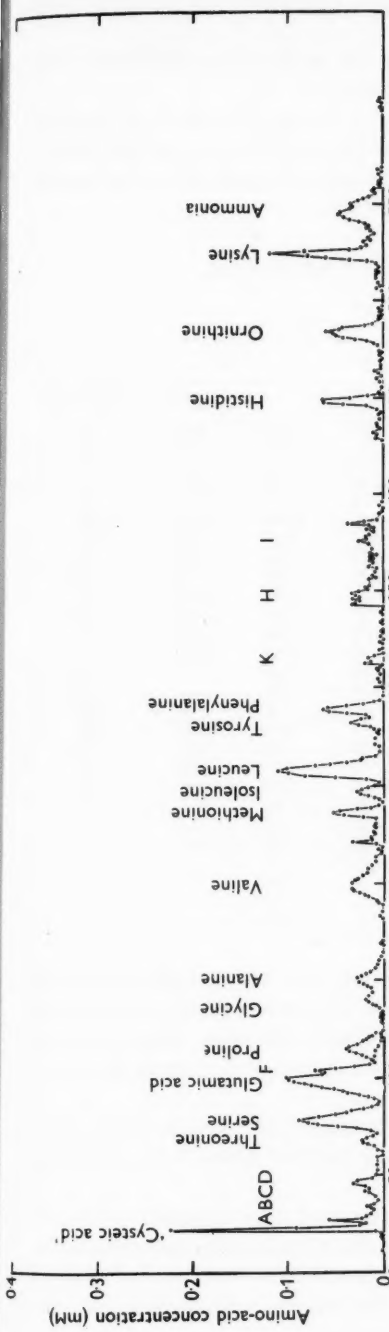

Fig. 4. 5-month-old cheese.

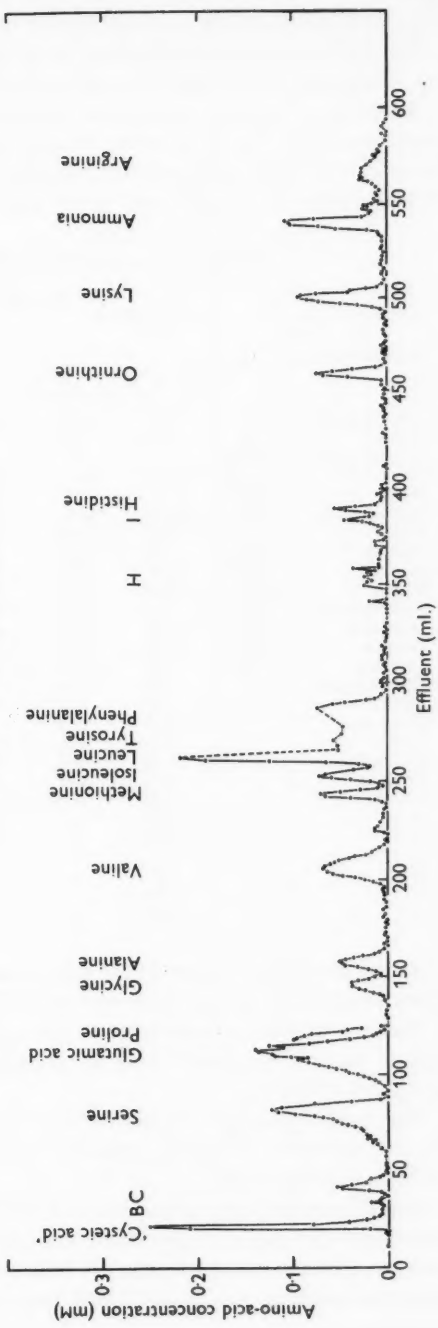

Fig. 5. 6-month-old cheese.

Figs. 4 and 5. Separation of amino-acids and peptides in the serum of Cheddar cheese (S1) on Dowex 50 resin.

ripening were data obtained for the basic amino-acids. For comparison the amino-acid content of casein (12) has been included in the table.

The recoveries of ninhydrin-positive material from the column were checked for two ripe cheese and were found to be 94·5 and 95% respectively.

A number of unknown peaks were encountered during the work; some, A–J, occurred most frequently. They were perhaps mainly due to peptides and have not yet been further investigated. In the early period of ripening (Fig. 1) the unknown peaks were so numerous that identification of amino-acid peaks was made difficult.

Table 1. *Amino-acids in the serum of Cheddar cheese*

(mg. ml.$^{-1}$ serum)

Cheese	E.D.2			S1				
Age (months) ...	18	6		3½	5	6		Casein, % composition
			% of total				% of total	
'Cysteic acid'	9·37	7·67	7·72	3·70	3·20	6·57	9·65	Nil
Cystine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0·4
Aspartic acid	9·80			Nil	Nil			7·0
Threonine	2·20	11·15	11·2	0·58	0·68	9·60	14·20	4·5
Serine	13·27			2·38	3·92			6·8
Glutamic acid	30·75	20·35	20·4	4·52	10·20	14·35	21·10	23·0
Proline	14·30	4·77	4·80	1·53	2·05	3·05	4·40	13·1
Glycine	4·06	2·26	2·27	0·42	0·50	1·23	1·80	2·1
Alanine	6·05	2·97	2·98	0·81	1·12	1·87	2·75	3·3
Valine	13·20	6·35	6·38	1·80	2·89	4·47	6·60	7·7
Methionine	2·92	2·85	2·87	1·42	1·70	2·59	3·81	3·5
Isoleucine	5·10	3·02	3·04	0·39	0·93	2·91	4·27	7·5
Leucine	9·75	13·00	13·06	3·25	5·70	10·30	14·90	10·0
Tyrosine	8·50	2·90	2·92	—	1·64	Lost	—	6·4
Phenylalanine	—	5·21	5·24	—	3·02	Lost	—	6·3
Histidine	—	1·40	1·41	—	1·80	1·56	2·30	3·2
Ornithine	—	2·11	2·12	—	1·91	2·44	3·30	Nil
Lysine	—	8·05	8·10	—	3·12	8·15	12·0	8·5
Ammonia	—	0·03	0·03	—	0·51	0·72	1·26	Nil
Arginine	—	4·92	4·94	—	5·87	6·15	9·05	4·2
A	—	0·12	—	0·70	Nil	0·16	—	—
B	—	0·35	—	0·36	0·31	0·25	—	—
C	—	2·35	—	0·33	0·37	0·15	—	—
G	—	—	—	—	0·65	—	—	—
H	—	1·11	—	—	—	1·70	—	—
I	—	0·39	—	—	—	0·70	—	—
J	—	0·93	—	—	—	1·00	—	—

Although excellent separation of aspartic acid, threonine, and serine could be obtained when a mixture of the pure acids was run on the column, there was in some cases difficulty in deciding whether aspartic acid or threonine was present in the serum (Figs. 5, 6), and in such cases a combined figure for aspartic acid and threonine and serine has been computed (Table 1).

The total amino-acid content of the serum of the ripe cheese amounted to about 75–100 mg. ml.$^{-1}$. This is in agreement with previously reported figures (6) for the serum of Emmenthaler cheese.

Identification of each amino-acid in the serum was deduced from the relative position and shape of its peak compared with those obtained when a mixture of known amino-acids was used. This procedure is generally reliable, but becomes suspect when applied to those ninhydrin-positive substances which run through the column with little or no ion-exchange.

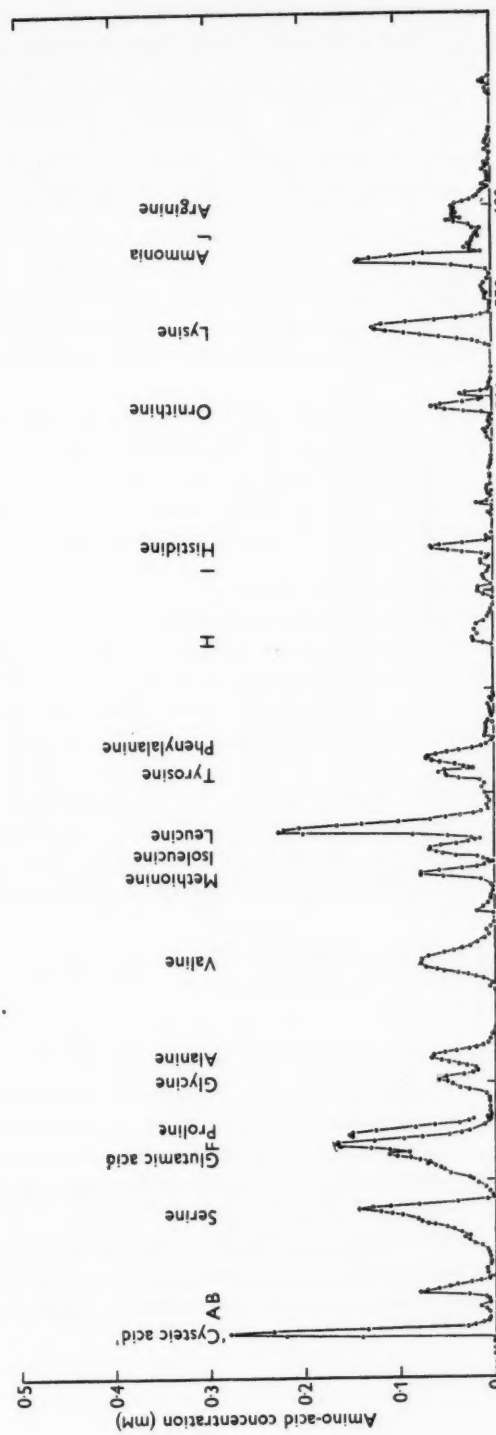

Fig. 6. Separation of amino-acids and peptides in serum of ripe Cheddar cheese on Dowex 50 resin. 6-month-old cheese (E.D.2).

Thus, the identity of the peak provisionally labelled 'cysteic acid' is in doubt. Its peak was, however, identical in shape and position with that of cysteic acid prepared by oxidation of L-cystine (13). Moreover, when cysteic acid was added to cheese serum and the mixture developed on the column only one enhanced peak with undisturbed symmetry was obtained. Nevertheless, in this position are to be found all the substances not adsorbed by the resin. There is, therefore, no real evidence concerning the identity of the 'cysteic acid' peak which may indeed contain a mixture of ninhydrin-positive substances.

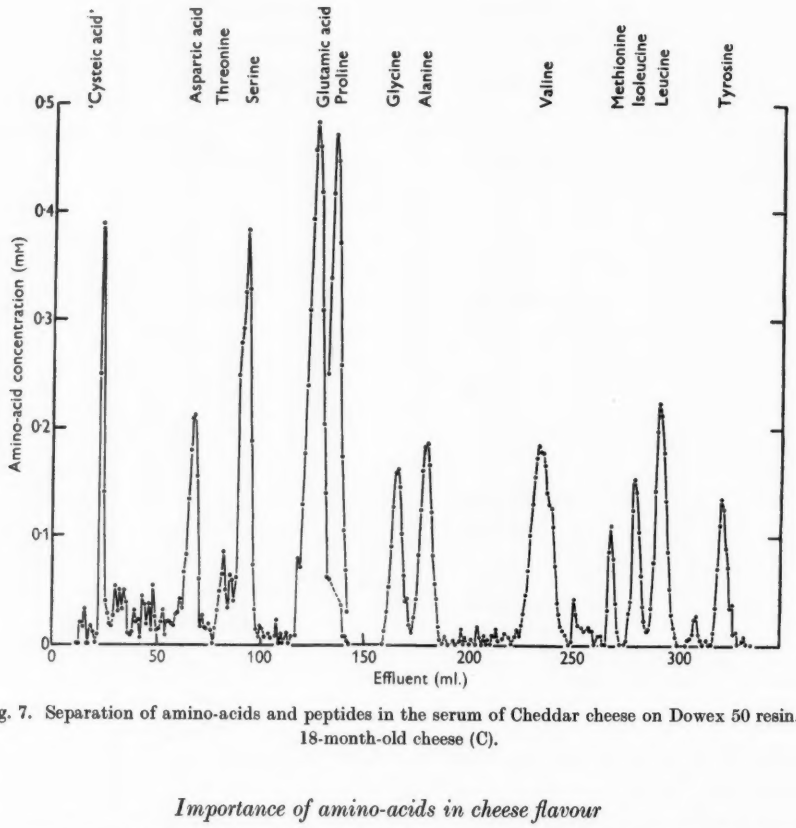

Fig. 7. Separation of amino-acids and peptides in the serum of Cheddar cheese on Dowex 50 resin. 18-month-old cheese (C).

Importance of amino-acids in cheese flavour

An aqueous solution of known amino-acids was prepared representing the average figures obtained from the two 6-month-old cheeses (Table 1); salt was added to give a concentration of 4·8% and the whole mixed with freshly made pressed cheese-curd. There was agreement among six workers, used to tasting cheese, that the flavour was not typical of Cheddar cheese; it was generally described as 'brothy'.

A mixture with very similar flavour could be prepared by adsorbing serum from ripe cheese on Dowex 50 (H+), washing with water to remove anions and non-ionic substances, eluting the amino-acids with ammonia solution and drying the eluate under vacuum to remove ammonia.

DISCUSSION

The ratios of the quantities of amino-acids found in the cheese serum were in some instances quite different from those found in casein. Thus, in cheese S1, there was much more lysine and arginine and less proline in the ripe cheese than would be anticipated from an analysis of casein. Ornithine, absent from casein, was also easily detected in the ripened cheese; it no doubt arose as a result of microbial synthesis. The presence of ornithine in hard cheese has been previously reported (14).

The peak provisionally designated as 'cysteic acid' is of interest because it shows a continual increase as ripening proceeds and in the mature cheese represents as much as 7–9% of the total ninhydrin-positive material in the serum. Calculation shows that for such a quantity of cysteic acid to be formed from cheese-protein the whole of the cystine content of the casein would have to be oxidized. It is hoped to make a later report on the identity of this peak.

Dacre (4) found that 'an amino-acid mixture corresponding to that found in a 6-month-old cheese gave no flavour to fresh curd'. The results reported here are in agreement with this statement only if 'flavour' means 'typical Cheddar flavour'. The brothy taste produced by a mixture of amino-acids similar to those found in ripe Cheddar cheese must provide a most important background flavour on which the typical flavour is superimposed.

SUMMARY

Quantitative data have been obtained concerning the amounts of individual amino-acids present in the aqueous phase of Cheddar cheese during the ripening process. They differed from the amounts found in casein by the larger amounts of basic amino-acids, smaller quantities of proline and the presence of ornithine.

Tasting tests showed that amino-acids contribute an important background flavour to Cheddar cheese.

An unidentified substance or group of substances, representing 7–9% of the total ninhydrin-positive material in the serum of ripe cheese, has been observed.

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REFERENCES

(1) TITTSLER, R. P., GEIB, D. S., SANDERS, G. P., WALTER, H. E., SAGER, O. S. & LOCHRY, H. R. (1946). *J. Bact.* **51**, 590.
(2) SHERWOOD, I. R. (1939). *J. Dairy Res.* **10**, 449.
(3) ALFORD, J. A. & FRAZIER, W. C. (1950). *J. Dairy Sci.* **33**, 115.
(4) DACRE, J. C. (1953). *J. Sci. Fd Agric.* **4**, 604.
(5) BAKER, R. J. & NELSON, F. E. (1949). *J. Dairy Sci.* **32**, 769.
(6) VIRTANEN, A. I. & KREULA, M. S. (1948). *Meijerit. Aikakausk.* **10**, 13.
(7) MULDER, H. (1952). *Ned. melk- en Zuiveltijdschr.* **6**, 157.
(8) HARPER, W. J. & SWANSON, A. M. (1949). *12th Int. Dairy Congr.* **2**, 147.
(9) KOSIKOWSKY, F. V. & DAHLBERG, A. C. (1954). *J. Dairy Sci.* **37**, 167.
(10) BARTHEL, C., SANDBERG, E. & HAGLUND, E. (1928). *Lait*, **8**, 285.
(11) MOORE, S. & STEIN, W. H. (1951). *J. biol. Chem.* **192**, 663.
(12) BLOCK, R. J. & BOLLING, D. (1951). *The Amino-Acid Composition of Proteins and Foods*, 2nd ed. p. 490. Springfield, Ill.: Thomas.
(13) GORTNER, R. A. & HOFFMAN, W. F. (1927). *J. biol. Chem.* **72**, 433.
(14) SIMONART, P. & MAYAUDON, J. (1952). *Ned. melk- en Zuiveltijdschr.* **6**, 1.

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REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION A. PHYSIOLOGY OF DAIRY CATTLE

THE BIOCHEMISTRY AND MICROBIOLOGY OF THE RUMEN

The previous review of this series by Owen (1) dealt with recent work on the physiology and biochemistry of the rumen that had been published up to the beginning of 1952. The main object of the present article is to review the work on the biochemistry and microbiology of the rumen that has appeared since that time. Nowadays, when so much is being published in this field, it cannot be claimed that every relevant paper has been cited, but it is hoped that a fair indication is given of the main advances that are being made.

Up till the late 1930's, when Krebs (2) published his review on the utilization of non-protein nitrogen by ruminants, progress in understanding the rumen processes was extremely slow. In the past 15 years, however, great advances have been made. The rapid progress in the earlier years of that period was reviewed by a group of workers in 1947 (3), and various aspects were dealt with by Phillipson (4) in 1946, by Elsden & Phillipson (5) in 1948, by Owen (1) in 1952 and also by Doetsch & Robinson (6) and Underwood & Moir (7) in 1953.

It is now well known that when the micro-organisms that abound in the rumen grow and multiply they convert cellulose and other carbohydrates to lower fatty acids, synthesize several of the vitamins and alter the nature of part of the nitrogenous constituents of the diet. Some of the products of this activity are absorbed into the blood stream through the rumen walls, but others pass further along the alimentary tract to be further digested and the products absorbed from the small intestine.

In the past year it has been shown by Dale, Stewart & Brody (8) that the temperature of the rumen ingesta varies very little, though it falls appreciably when the animal drinks. It tends to rise slightly to about 40° C. and then to decline again to the normal body temperature of the animal as the time after the previous meal increases. The pH of the ingesta remains between 6 and 7, partly as a result of the absorption and passing on of the end-products of digestion and partly as a result of the flow of saliva into the rumen. Two of the factors which influence the pH have been shown by Ammerman & Thomas (9) and Cason, Ruby & Stallcup (10) to be the amount of fermentable carbohydrate in the diet and the amount and nature of the ash content of the ingesta.

RUMINANT SALIVA

One of the main fluids that influence rumen digestion and help to provide a suitable medium for the micro-organisms is the saliva. It is secreted in very large quantities, as much as 50 l. per day in the cow (11), and has been shown by Nasr (12) to contain no amylase. For an account of the early work on saliva the review by Mangold (13) should be consulted.

McDougal (14) analysed parotid and mixed saliva obtained from the sheep and gave the average composition of the two types. He concluded that the parotid glands were the main source of ruminant saliva. From his results a formula for a synthetic saliva was derived, suitable for use as a medium in the study of rumen bacteria. McDougal's paper included a comparison of mixed sheep saliva with human saliva and of sheep parotid saliva with sheep blood serum.

The phosphorus content of saliva has been further commented on very recently by Clark (15), who concluded that there is a circulation of soluble phosphorus from the blood to the saliva thence to the rumen and then back to the blood through the small intestine. His figures for goats and cattle obtained in South Africa of 8·5 and 9·6 mg. P/100 ml. are much lower than that of 68·5 mg. P/100 ml. found by McDougal (14) for sheep in Britain. The high level of phosphorus in rumen ingesta was suggested by Clark to be maintained by this circulatory mechanism, so that it was not directly dependent on the phosphorus content of the diet or the blood.

RUMEN GASES

As already indicated, the rumen micro-organisms are bathed in a medium derived in large measure from the saliva. They are, moreover, maintained under anaerobic conditions partly as a result of the gases produced in the course of their own metabolism. It is with these gases that this section of the review is concerned. A comprehensive account of the earlier work done on rumen gases was published in 1945 by Cole, Huffman, Kleiber, Olson & Schalk (16) where special attention was given to the problem of bloat. Analysis (17) showed that a typical sample consisted of 67% CO_2, 26% methane, 6% nitrogen, 0·1% hydrogen sulphide and less than 1% oxygen. It is proposed to consider in turn the sources of each of the gases starting with CO_2. Only reactions which are considered likely by the author to occur in rumen liquid are discussed, and it is not suggested that the list is comprehensive, but simply that it is indicative of what may take place.

Carbon dioxide. In certain systems dismutation of two molecules of pyruvic acid has been shown to give rise to one molecule each of lactic and acetic acids and CO_2. With *Propionibacteria* the overall reaction shown below has been observed to occur.

$$\underset{\text{lactic acid}}{3CH_3.CHOH.COOH} \rightarrow \underset{\text{propionic acid}}{2CH_3.CH_2.COOH} + \underset{\text{acetic acid}}{CH_3COOH} + CO_2 + H_2O$$

Others that are known to take place are decarboxylation reactions such as that giving rise to acetic acid and CO_2 from pyruvic acid, or that giving pyruvic acid and CO_2 from oxaloacetic acid. Stickland reactions between amino-acids such as alanine and glycine may also occur and would produce both ammonia and CO_2 as in the following example:

$$\underset{\text{alanine}}{CH_3CHNH_2COOH} + \underset{\text{glycine}}{2NH_2CH_2COOH} + 2H_2O = 3CH_3COOH + 3NH_3 + CO_2$$

These reactions have been discussed by Stephenson (18), and more recently by Nisman (19).

The exact processes by which CO_2 arises from glucose and some other carbohydrates in the rumen have not yet been fully elucidated, but it seems likely that the main pathway may be glucose → glucose-6-phosphate → fructose-6-phosphate → fructose 1:6-diphosphate → 'triose phosphate' → 1:3-diphosphoglyceraldehyde → 1:3-diphosphoglyceric acid → 3-phosphoglyceric acid → 2-phosphoglyceric acid → phospho-enol-pyruvic acid → pyruvic acid. This sequence of reactions, which is often known as the Embden, Meyerhof & Parnas system (18), is known to be energy-yielding, and it will be noticed that down to the pyruvic acid stage no CO_2 is split off. Pyruvic acid, however, can give rise to CO_2 by several reactions including decarboxylation to acetic acid, the latter being a well-known product of fermentation in the rumen. In addition to the above scheme, the possibility exists that degradation of glucose may give rise to 6-phosphogluconic acid and that this is then decarboxylated to pentose-phosphate through the intermediate 2-keto-6-phosphogluconic acid. Further, a degradation of glucose via 2-keto gluconic acid without the necessity for phosphorylation has been postulated. Whether any of these pathways is the one most pertaining in rumen liquid still remains to be shown. The anaerobic nature of rumen contents makes it appear that the usual Embden, Meyerhof & Parnas system may prove to be the main degradation pathway of sugars under normal conditions, the one point against this being the very high concentration of fluoride required to inhibit bacterial activity in rumen liquid. Further work is required with washed bacterial suspensions.

Substances other than sugars and pyruvic acid can participate in exchanges of CO_2 in the presence of rumen bacteria. Thus Johns (20) in 1951, using *Veillonella gazogenes*, isolated from the rumen of sheep, showed that succinic acid was decarboxylated to yield propionic acid and CO_2 (p. 243), and in 1954 the work of Sirotnak, Doetsch, Robinson & Shaw (21) indicated that aspartic acid could be converted to succinic acid which was then decarboxylated to give propionic acid. Shaw and his colleagues (22) have shown that bacterial suspensions, prepared by removing the protozoa by centrifugation and suspending the bacteria in phosphate buffer, produced large amounts of CO_2, not only from maltose and pyruvate but also from succinate, fumarate, malate and formate.

A further source of CO_2 has been discussed by Masson & Phillipson (23), who have demonstrated that during the absorption of volatile fatty acids through the rumen wall, chloride and bicarbonate ions pass into the rumen from the blood. The saliva is also rich in carbonate, so that the CO_2 of the rumen can arise in many different ways. It has now been shown that in addition to producing CO_2 rumen bacteria are capable also of utilizing the gas. This subject has been studied very recently by Huhtanen, Carleton & Roberts (24), using $^{14}CO_2$ and bacteria isolated by Gall & Huhtanen (25, 26). They found that when $^{14}CO_2$ was present the lactate utilizer (designated RO-C_1 by Gall & Huhtanen) produced volatile fatty acids which contained ^{14}C, and that the lactic acid producing bacteria (RO–L5, RO–CR and RO–L_1) showed a great tendency to assimilate the radioactive CO_2. On investigating the incorporation of the ^{14}C from $^{14}CO_2$ into the protoplasm of RO–L5 it was found that 35–40% of the activity was accounted for in the 'nucleic acid' extract, the remainder being in the 'protein' fraction. Most of the activity in the protein fraction was in the five amino-acids, aspartic acid, glutamic acid, threonine, arginine and phenylalanine.

Methane. The production of methane by bacteria has been widely studied and has been reviewed by Stephenson (18). In the last two or three years production of methane in the rumen or by rumen bacteria has been studied by Beijer (27), Doetsch, Robinson, Brown & Shaw (22), Doetsch, Robinson & Shaw (28), McNeill & Brown (29) and Davis & Squires (30).

In Beijer's work 1% sodium acetate, propionate, butyrate or succinate was added to flasks completely filled with rumen liquid from a goat, and the mixture incubated for 4–6 hr. at 40° C. The gas produced was analysed and compared with the gas obtained from control flasks containing rumen liquid without any addition. The results showed that acetate, propionate and butyrate were not fermented by the rumen bacteria. Formate, however, proved an excellent source of methane, 1 molecular equivalent of formate giving 0·20 molecular equivalent of methane compared with the theoretical figure of 0·25 which would be expected if the reaction went according to the following equation:

$$4H.COOH = CH_4 + 3CO_2 + 2H_2O.$$

In view of what has already been said about the formation of CO_2 it is interesting to note that this reaction would also result in a large yield of that gas in addition to methane. Succinic acid was also found to give rise to an appreciable amount of methane. In contrast to the results obtained by Beijer with goat rumen liquid, Doetsch *et al.* (22), using suspensions of bovine rumen bacteria, found that fermentation of formate gave rise to equal volumes of CO_2 and some other gas which they stated to be hydrogen (28). This would imply the presence of a formic hydrogenlyase system catalysing the reversible reaction

$$H.COOH \rightleftharpoons CO_2 + H_2.$$

McNeill & Brown (29) tested glucose, maltose, cellobiose, pyruvate, lactate, propionate, acetate, formate, succinate and fumarate as substrates with suspensions of bovine rumen bacteria, and came to the conclusion that formate yielded much greater quantities of methane than any of the other substrates. It seems, therefore, that formic acid is likely to be one of the main sources of methane in the rumen. Formate itself has been shown to arise in some bacteria from a hydrolytic cleavage of pyruvic acid resulting in a mixture of acetic and formic acids, but the presence of such a system in rumen bacteria remains to be shown. If such a reaction does take place it would seem to do so only slowly, since a rapid conversion along these lines would mean that pyruvic acid would be as good a source of methane as formic acid itself. Davis & Squires (30) have shown with the mass spectrometer that during anaerobic fermentation of paper by the micro-organisms of cowdung, ethane, ethylene, propane and propylene are all produced in small amounts, but the major constituents of the mixture of gases are CO_2 and methane. It was found also that small amounts of ethane and ethylene were formed when ethanol, acetate, butyrate and glucose were fermented, and that acetylene could be detected at the intermediate stages of glucose fermentation and when acetate and butyrate were being fermented. Propylene was found in the early stages of butyrate and glucose fermentations but not in the later stages. It would be most valuable if similar studies could be made with rumen liquid or with bacterial suspensions from the rumen.

Other gases. Little is known about the precursors of the gases, other than CO_2 and methane, which are formed in the rumen. As has already been pointed out, formic acid has been suggested as one possible source of hydrogen, and as indicated in a short review of the subject published by Cole *et al.* in 1954 (16) it is well known that with some bacteria, hydrogen may be formed in the presence of pyruvate, fumarate, malate, certain sugars and some amino-acids. The formation of hydrogen sulphide in the rumen has also been briefly reviewed by Cole *et al.*, and perhaps one of the most important recent contributions to this problem is that of Lewis (31), who has shown that sulphate is reduced to sulphide in the rumen of sheep and also *in vitro* by washed suspensions of rumen bacteria. With hydrogen gas as hydrogen donor the optimum pH was about 6·5. Sulphite and thiosulphate were also reduced to sulphide using gaseous hydrogen or a hydrogen donor such as formate, pyruvate, fructose, glucose, lactate, succinate, ethanol, citrate or malate.

RUMEN BACTERIA

The study of rumen bacteria, both in pure culture and in mixed suspensions, has made great advances in recent years, but there is still much to be done. It is probably too early yet to expect that much should be known on the interactive effects of different types of rumen micro-organisms living in association as in the rumen itself, but it is hoped that in due course it may be practicable to apply to rumen bacteria an approach such as that made recently by Rosebury, Gale & Taylor (32) in their study of the micro-organisms indigenous to man. Already studies have been made by Gyllenberg (33), in Sweden, of some of the interactive effects in cellulose breakdown. Gyllenberg has found that the cellulolytic activity of clostridia appears to be increased in the presence of Streptomyces.

For a review of fairly recent work on rumen bacteria up to the early part of 1952 the reader is referred to an article by Doetsch & Robinson (6). The papers cited here have in the main been published since that time.

Moir & Masson (34) published in 1952 an illustrated scheme for the microscopic identification of the rumen micro-organisms of the sheep, and in it they attempted to produce a system of numbering to identify various bacterial types, instead of using the binomial system for which they said sufficient evidence was often unavailable. Thirty-three types, many shown in photomicrographs, were described with respect to shape, size, Gram and iodine staining. In addition, some observations on motility, formation of spores and other relevant properties were recorded. It was pointed out that no technique to enumerate the fixed bacteria responsible for cellulose and starch catabolism had been devised. The scheme put forward by Moir & Masson would seem to afford a method of quantitative evaluation of a bacterial population through differential counts, and might provide a useful control in many studies of a biochemical nature. Heald, Krogh, Mann, Appleby, Masson & Oxford (35) have devised a direct method for making viable counts of facultative anaerobes, and have reached the conclusion that facultative anaerobes play a more important role in rumen metabolism than had been previously believed, this early lack in recognizing their importance being due to a concentration of attention on the strictly anaerobic process of cellulose digestion. Mann, Masson & Oxford (36), in a further study of the facultative anaerobes of the previous paper, have shown that of 120 streptococcal isolates 82% showed fermentation and other reactions similar to those of *Streptococcus bovis*, 6% resembled *Strep. faecalis* and the rest (12%) were unclassifiable. The large, Gram-negative sarcina-like organism (no. 26 in Moir & Masson's scheme) which was noted by earlier workers was also isolated in pure culture and named *Sarcina bakeri*.

The occurrence of facultative anaerobes has also been the subject of a paper by Higginbottom & Wheater (37), who investigated the incidence of *Strep. bovis* in cattle, and have shown that in a fistulated heifer and a fistulated steer the numbers remained relatively constant at 10^5–10^7/ml. over a period of 3 years. It was found that the numbers were little affected by changes in diet from stall- to grass-feeding or vice versa.

Lactate-utilizing organisms from the bovine rumen have been studied by Gutierrez (38). He found that lactate utilizers could be observed only after several days of incubation, the first to

appear being said to belong to the genus *Desulphovibrio* but this organism was not present in sufficient numbers (only 50,000–100,000/ml.) to be considered of prime importance, though it was suggested that it might be responsible for the production of toxic amounts of hydrogen sulphide under conditions in which the animal was ingesting relatively large amounts of sulphate. After 7–14 days' incubation other lactate-utilizing bacteria began to appear, and eventually twenty-four strains were isolated; they resembled *Propionibacterium acnes* but utilized lactate. The numbers of these organisms were found to vary from 250,000 to 7×10^9 with an average of $1{\cdot}3 \times 10^9$, and in view of these high counts a search for the bacterium was made in hay which proved to contain large numbers of organisms of a similar type. The importance of such organisms in the rumen has yet to be assessed. *Veillonella gazogenes* (39) was also found, but in small numbers.

Bryant & Burkey (40) have recently made a detailed study of cultural methods for rumen bacteria, and have isolated 896 strains, all of which were strict anaerobes except the facultative anaerobes of the genus *Streptococcus*. The morphological types cultured included cocci, rod-shaped organisms, spirilla and spirochetes. Spores were not observed in any of the cultures; many strains produced a considerable amount of capsular material. Their isolates included cellulolytic rods which had the property of forming rosette-shaped bundles closely resembling the rosettes observed by various other workers (Baker (41); Baker, Nasr, Morrice & Bruce (42); Moir & Masson (34)). A comparison of various techniques used in cultural investigations of rumen bacteria has been made by Doetsch, Robinson & Shaw (43) who found in dilutions of 10^{-8} or 10^{-9} a capsulated diplococcus and a long, thin, rod-shaped bacterium. Huhtanen & Gall (44) isolated five types of small, thin, curved, motile rods, all of which were active fibre digesters, and in addition they isolated three larger, short, straight rods occurring in chains. They appeared to be important and were non-spore-forming obligate anaerobes producing butyric, acetic and lactic acids.

Several new studies have been reported in the last few years on the nutritional requirements of rumen bacteria. It has usually been observed in these studies that for maximum numbers of rumen bacteria to grow, rumen liquor must be included in the growth medium. McNeill, Doetsch & Robinson (45) have stated in a brief abstract that the factors in rumen contents which appear to be essential for the bacteria are stable to acid and alkaline hydrolysis and can be extracted with activated adsorbing agents. They found also that they were not completely replaceable by peptones, meat extract, yeast extract, liver extract, feed infusions, various amino-acids or casein hydrolysates. Doetsch and his colleagues were not able to identify these essential factors, but they concluded that they are not present in the rich nitrogenous media usually employed in cultural investigations (46).

Wasserman, Seeley & Loosli (47) isolated an organism from rumen liquid which was found to be a variant of the species *Lactobacillus bifidus*. The majority of the strains required or were stimulated by vitamins of the B-complex. The same authors (48) found that some isolates required a factor or factors which they have not identified but which is supplied by tomato juice, grass juice, distillers' dried yeast, molasses solubles and bovine faeces, and other less potent sources which include rumen liquor.

Work reported by Bentley, Lehmkuhl, Johnson, Hershberger & Moxon (49) suggests that one of the unknown factors which stimulate the rate of cellulose digestion by rumen micro-organisms is a steam-volatile fatty acid. Acetic, propionic and butyric acids were inactive in this respect, but activity was observed with caproic, valeric and to a smaller degree with *iso*butyric and *iso*-valeric acids. Bryant & Doetsch (50) have also reported the presence of a growth factor in the volatile acid fractions of rumen fluid. These papers are discussed in more detail in the section dealing with cellulose digestion (p. 239). Whether these volatile fatty acid constituents are also the factors required by the Lactobacilli described by Wassermann *et al.* is not yet known.

The diet of the animal has been known for a long time to affect the numbers and types of bacteria to be found in the rumen. Wilson & Briggs (51) have developed a method of assessing the number of viable organisms per g. of bovine rumen material which has given counts of 10^{10} and 10^{11} organisms per g. They have used the method to study variations from cow to cow, from day to day and from hour to hour. The effect of dietary changes on the viable count was investigated

in cows receiving rations which consisted of hay *ad lib.*, 6 lb. hay with 12 lb. concentrates and 16 lb. hay with 8 lb. concentrates. Periods while the animals were grazing were also included. Considering how much the diets differed, the counts showed little change. The effect of fasting has been studied by Quin, Oyaert & Clark (52) who found that after 48 hr. a noticeable depression had occurred in the ability of rumen bacteria to ferment sugars and digest cellulose. Gall, Thomas, Loosli & Huhtanen (53) fed lambs on purified diets and took stomach-tube samples for bacteriological studies. They concluded that when urea was fed in the absence of sulphur, the bacteria were almost all facultative anaerobes rather than the obligate anaerobes usually found in the rumen. The animals did badly on this ration and throve better when sulphur was included. When the animals were fed casein, the types of bacteria were found to differ from those predominating when other purified diets were fed. Gall, Huhtanen, Saunders & Schmidt (54) found that two basically different types of feed, high grain rations and high roughage rations, each produced its own characteristic rumen flora. High roughage rations encouraged delicate obligate anaerobes which acted on fibre to produce butyric, propionic and acetic acids. In contrast, a high grain ration encouraged bacteria of a less strictly anaerobic type most of which, in the artificial rumen, acted on readily available carbohydrate to give lactic acid but had relatively little action on fibre. When combinations of these two rations were fed the type of rumen flora fell between the two extremes. Bryant & Burkey (55) have shown that the number of cellulose-digesting organisms tends to vary with the crude fibre content of the diet. They found that starch-splitters were lowest on a ration of straw and highest on concentrates. The same authors (56) isolated substantially the same predominant groups of bacteria from hay-fed and silage-fed heifers and stated that none of the bacteria found in alfalfa silage was present in the high dilutions of rumen contents cultured. Williams, Nottle, Moir & Underwood (57) found the numbers of micro-organisms in the rumen to be markedly affected by the levels of both dietary protein and dietary starch, the extent to which either was effective depending on the proportion of the other present. On a low-protein diet, increased starch ingestion greatly reduced bacterial counts and caused marked changes in the types present. On diets giving high or medium starch intakes, increased protein levels gave significant increases in bacterial counts, no such effect being evident on a low-starch diet.

Two other papers remain to be considered in this connection. Hungate, Dougherty, Bryant & Cello (58), in a study of acute acid indigestion in sheep, induced by an abrupt change from a hay to a grain diet, found that different types of micro-organisms were present in different ratios before and after feeding the grain, an increase occurring in Gram-positive organisms, e.g. rods and cocci. In an experiment in which glucose was fed, Gram-positive cocci and diplococci increased in one sheep, while in a second sheep protozoa were absent. The cocci isolated proved to be *Streptococcus bovis*, and the conclusion was reached that this organism was the main agent in the production of the increased acidity. Scarisbrick (59) studied acid indigestion in sheep caused by feeding mangolds and gives a list of some of the mechanisms operating to preserve the neutrality of the rumen when there is a tendency for excessive production of acid; no bacteriological investigation was made.

The chemical composition of rumen bacteria has been studied by a number of workers. Johansen, Moir & Underwood (60) analysed rumen bacteria for sulphur-containing amino-acids, and found that the nitrogen of cystine plus cysteine and of methionine accounted for 1·63 and 1·18% respectively of the total nitrogen when the sheep were on 'dry' feed, and for 1·50 and 1·54% when they were on 'green' feeds. The difference between the two values for methionine was significant, but not the difference between the values for cystine plus cysteine. Holmes, Moir & Underwood (61) used quantitative paper chromatography to analyse rumen bacteria for fifteen amino-acids. The samples of bacteria were again prepared from sheep on 'dry' and 'green' feeds. The two samples of bacteria had very similar amino-acid distributions and showed no outstanding differences from those recorded in the literature for many typical micro-organisms. Four species of bovine rumen bacteria have been shown by Tsuda (62) to contain sixteen or seventeen amino-acids including all the essential ones. Synge (63) has found diaminopimelic acid to be present in

Ruminococcus flavefaciens (strain S), and also in a coliform xylose fermenter and in *Clostridium butyricum* and *Cl. welchii* from sheep rumen, but not in *Streptococcus faecalis* or in four strains of amylolytic streptococci. Synge (64) has suggested that diaminopimelic acid may be required as a growth factor by *Ruminococcus flavefaciens*. Other amino-acid analyses have been reported by Duncan, Agrawala, Huffman & Luecke (65) and by Loosli, Williams, Thomas, Ferris & Maynard (66), but their results are discussed more fully in the section of the present review which deals with protein and amino-acid synthesis (p. 244). Encapsulation in rumen bacteria has been studied by Hobson & MacPherson (67) and by Greenwood (68). Davidson (69) has studied pigmentation in microbial fractions from sheep's rumen and has found that pigmentation in bacteria from rumen of hay-fed sheep is caused by pigments normally associated with plant chloroplasts; no chlorophyll typical of the known pigmented bacteria was found.

The enzymes of rumen micro-organisms have not been studied in any great detail, but from time to time papers have appeared on various aspects of the subject. Antoniani (70) found that the cellobiase in rumen liquid was not exclusively derived from bacteria, part of it at least being of vegetable origin. Hobson & MacPherson (71) made a study of amylases isolated from sheep rumen strains of *Clostridium butyricum* and from a *Streptococcus* grown in media containing dissolved starch. The enzymes were found to be α-amylases with no well-defined pH optima. The branch linkages in amylopectin were found to act as obstructions to both enzymes. Maltose and maltotriose with some glucose were the main end-products of the hydrolysis of amylose by these enzymes. Neither of the enzymes attacked these sugars further, but both hydrolysed maltotetrose. Karunairatnam & Levvy (72) and Marsh (73) have studied a glucuronide-decomposing enzyme prepared from rumen micro-organisms. They found that it had a pH optimum of between 6·1 and 6·6, depending on the purity of the preparation, and that it was associated with an organism occurring in the rumen of the sheep. It was said that the natural substrate for this enzyme was not a conjugated glucuronide such as is found in urine, but a plant polysaccharide containing uronic acid residues or one of their fission products. Conchie (74) has isolated a β-glucosidase of essentially microbial origin as a cell-free extract from sheep rumen contents. It had two pH optima 5·4 and 5·8 and was inhibited by glucono-1:4-lactone and glucono-1:5-lactone. Sym (75) and Kimura & Umezu (76) have studied the proteolytic activity of rumen contents. The latter authors claim to have demonstrated the presence of a proteinase. In their work, for which they used the rumen contents of the goat and the camel, short-term incubations showed the presence of acid and alkaline phosphatases and slight amylase activity; urease and lipase activity were found only after prolonged incubation (2 days). It is strange that in this work no urease activity was found, since the conversion of urea to ammonia in rumen contents has frequently been demonstrated; e.g. Pearson & Smith (77) found that 100 g. rumen liquid could convert 100 mg. urea to ammonia in 1 hr. The presence of other enzymes concerned in nitrogen metabolism in the rumen has been studied by Rodwell (78, 79), who has found amino-acid decarboxylase in bacteria isolated from sheep rumen contents. The enzymes in the strains from sheep rumen included histidine and arginine decarboxylases, while from the horse stomach, strains were isolated which decarboxylated arginine, histidine, lysine and ornithine.

RUMEN PROTOZOA

Work on the protozoa of the rumen has been making substantial progress in the past two or three years. Exceedingly helpful and well-illustrated accounts of these micro-organisms were published as long ago as 1927 by Becker & Talbott (80), and in 1929 by Mangold (13). In recent work on the subject cultural studies have been made by Sugden & Oxford (81) with holotrich ciliates, and by Sugden (82) with oligotrich ciliates. The former showed that the survival time of the holotricha was increased 4–5 days by glucose and cellobiose, by 2–3 days by fructose, galactose, sucrose, raffinose, inulin, bacterial levan and salicin and by 1 day by melibiose. Many other substances, such as xylose, ribose, maltose, lactose and lower fatty acids, were tested but were found to have no effect, while the presence of mannose, glucosamine or galactosamine greatly shortened the survival time. The mineral requirements of the protozoa were also studied and it

was suggested that some 'trace' metal was needed for their survival. Sugden (82) has described a method for the separation of the large oligotrich protozoa. Cultures of *Metadinium medium* organisms survived for as long as 12 days in a medium of powdered cotton-wool and hay, while species of *Entodinium* survived for 12–14 days under conditions that were similar except that the carbohydrate used was rice starch instead of cellulose. It was shown that *Metadinium medium* lost the ability to utilize cellulose in the absence of bacteria, so that it probably does not contain a cellulase of its own but relies on symbiotic cellulolytic bacteria. Streptomycin was not toxic to these protozoa. Oxford (83) found that holotrich ciliates, when incubated with glucose, fructose or sucrose, gave storage of microscopic granules of a glucosan which was practically free from protein, while other substances tested, glucuronolactone, galatose, mannose, xylose, arabinose, sorbose, lactose, cellobiose and maltose, were not used for any marked polysaccharide synthesis. In an extension of this work Masson & Oxford (84) showed that glucose, fructose, sucrose, inulin, bacterial levan, and to a lesser extent cellobiose, were utilized for rapid and extensive storage of iodophilic polysaccharide granules. The ability of the protozoa to store glucosan has been said by Heald & Oxford (85) to be of twofold significance to the host animal, in that it prevents the rumen bacteria from fermenting all the soluble carbohydrate present in the rumen, while endogenous breakdown of the glucosan releases a steady flow of metabolites into the rumen. The polysaccharide produced by the holotrich ciliates of the sheep's rumen have been shown by Forsyth & Hirst (86) to have a highly branched molecular structure closely similar to that of amylopectin. There is one terminal glucose residue for every twenty-two glucose residues in the molecule. A very similar polysaccharide obtained from *Cycloposthium* cells from the caecum and colon of the horse has been described by Forsyth, Hirst & Oxford (87). The fermentations of the genera *Isotricha* and *Dasytricha* have been studied by Heald & Oxford (85), who have shown that these protozoa are resistant to streptomycin and can ferment glucose, fructose, sucrose, raffinose, inulin, rye-grass levan and to a lesser extent cellobiose. The products of glucose fermentation have been shown to be lactic acid, acetic acid, propionic acid (traces), butyric acid, CO_2 and hydrogen. Soluble nitrogenous compounds which were not ammonia or amino-acids were also produced. Chemical studies on the rumen protozoa of the goat have been made by Kameoka, Takajasji & Morimoro (88) who, from the evidence of two-dimensional paper chromatograms, reached the conclusion that all the essential amino-acids except methionine were present, while of the non-essential amino-acids only serine appeared to be absent. These findings, in the opinion of the reviewer, require careful re-investigation since it appears unlikely that a mixture of amino-acids obtained from a source such as protozoa would be completely lacking in two amino-acids which are so commonly found as serine and methionine. In the same work Kameoka *et al.* have shown that on a diet free from starch the numbers of protozoa present in the rumen were considerably reduced, and that the starch-like granules they normally contain rapidly disappeared.

The biological value of a sample of dried protozoa has been determined by McNaught, Owen, Henry & Kon (89) with rats. It was found to be 80. This did not differ significantly from the biological value of rumen bacteria which was found in this same work to be 81, compared with 88 found by McNaught, Smith, Henry & Kon (90) and 79 found by Reed, Moir & Underwood (91). There was, however, a marked difference in the true digestibility of the nitrogen in the dried protozoa as compared with that in the dried bacteria, the values being 91 and 74% respectively. Thus the conversion of bacterial protein to protozoal protein by the protozoa in the rumen, a process which has been postulated by some workers as being one of the functions of the protozoa, would render the bacterial protein more digestible to the host.

D'Agostino (92) has studied the behaviour of protozoa from the rumen of goats in the presence of certain vitamins. In this particular paper he reported that vitamin K shortened the survival time of the protozoa.

CELLULOSE DIGESTION

In recent years much attention has been paid to the fact that the presence of some unidentified compound or compounds in rumen liquid has the effect of stimulating cellulose digestion. MacLeod & Brumwell (93) tested the effect of various by-products of the fishing industry, and

showed that several of these, such as whale and herring solubles and halibut muscle hydrolysate, stimulated hydrolysis, while the ash of whale solubles and a mixture of eight water-soluble vitamins, cysteine and methionine showed no ability to promote fermentation. Since common salt is used to quite a large extent in supplementing cattle foods in certain parts of the United States, Cardon (94) has studied the effect on cellulose digestion of an increased salt concentration in rumen fluid. In the amounts that would be given in high salt rations, salt did not appear to cause any decrease in rumen microbial activity or in the amount of cellulose digested. *In vitro* experiments confirmed these findings, and addition of phosphate buffer was also found to be without effect. The effect of minerals on cellulose decomposition has been investigated in artificial rumens by Burroughs *et al.* (95), who found that phosphorus and iron were effective in stimulating cellulose digestion. Brooks, Garner, Gehrke, Muhrer & Pfander (96) found that the presence of maize oil decreased the digestion of cellulose *in vitro* by 40–94%. Maize oil or lard also significantly decreased cellulose digestion in the rumen of sheep. The depressing effects of fat were partly overcome by the addition of alfalfa ash. An *in vitro* stimulation of cellulose digestion by stilboestrol has been shown by Brooks, Garner, Muhrer & Pfander (97); *in vivo*, however, it was found that wethers could not tolerate the levels of 10 or 20 p.p.m. that were used. In the same work cholesterol and oestrone also appeared to increase cellulose digestion *in vitro*.

Garner, Muhrer, Ellis & Pfander (98) claim that they have prepared extracts from rumen contents which contain a factor which stimulates the growth and cellulolytic activity of rumen micro-organisms and inhibits the growth of micro-organisms said to be undesirable contaminants in the digestive tracts of ruminants, but details of the work are not yet available. Experiments have been made by Hall, Cheng & Burroughs (99) to study the presence or absence of stimulatory factors in feedingstuffs. In continuation of that work (100), using washed suspensions of rumen bacteria, various combinations of B vitamins and small amounts of other substances were tested. A combination of vitamin B_{12} and biotin seemed particularly stimulatory, and slight stimulation was observed also with ribose, L-sorbose, xylose, arabinose and rhamnose. A search for factors that increase cellulose digestion has also been made by Bentley and his colleagues (101, 102, 103) who have shown that the factors could be adsorbed on to and eluted from charcoal, and were stable to autoclaving in acid or alkaline solution. In the last of these three papers it is reported that with starch as a source of energy, cellulose digestion decreased though synthesis of riboflavin, niacin, pantothenic acid and B_{12} increased. With less starch, cellulose digestion improved. As already mentioned, Bentley *et al.* (49) found that acid distillates of rumen liquid possessed stimulatory properties, suggesting that the principle was contained in the steam-volatile fatty acid fraction. Caproic acid and valeric acid were found to be most active, while *iso*valeric and *iso*butyric were also effective. Acetic, propionic, butyric and straight chain C_7 and C_{10} acids were inactive. Apparently a non-volatile stimulator also exists which might be related to the amino-acid content of feedingstuffs, and preliminary studies have suggested that α-amino derivatives of the volatile acids were active. It was pointed out that the activity of the volatile fatty acids and amino-acid precursors was not restricted to cellulose digestion, but that conversion of non-protein nitrogen to protein was also stimulated. Bryant & Doetsch (50) have shown that *Bacteroides succinogenes* gave good growth when combinations of two of the acids *iso*butyric, *n*-valeric, *iso*-valeric, DL-α-methyl-*n*-butyric or *n*-caproic acids were tested, provided one of the acids in the combinations was a straight-chain acid and the other a branched-chain acid. Since *Bacteroides succinogenes* is believed to be an important cellulose digester in the rumen the importance of this finding needs no stressing.

Much interest has been focused in recent years on the effect of antibiotics and chemotherapeutic agents on live-weight gains and efficiency of food utilization by farm animals, and many experiments have been done to test the effect of such compounds on cellulose digestion. Oyaert, Quin & Clark (104) found that sulphanilamide in concentrations of 0·5 to 1·0 part per 1000 of rumen ingesta depressed cellulose digestion and appetite. The fermentation of sugar and the formation of gas were also suppressed but only by the higher concentrations. Turner & Hodgetts (105) ound that on feeding about 25 mg. aureomycin hydrochloride per kg. body weight to sheep the

rumen flora appeared to be reduced by some 75%, the survivors being mainly Gram-positive rods and cocci; fermentation was greatly depressed, administration of rumen liquor and yeast extract restoring normal conditions. Various effects of aureomycin have been studied by Chance and his associates (106, 107, 108). Their results indicated that an increase occurred in the rate of removal of dry matter, crude fibre, crude protein and nitrogen-free extract when the antibiotic was fed, and that the increase was directly related to an increase in the coliform population. In the same work the presence of aureomycin was believed to retard the synthesis of nicotinic acid and pantothenic acid.

Wasserman, Duncan, Churchill & Huffman (109) showed that at low concentration penicillin actually stimulated cellulose digestion by rumen bacteria, and that neomycin and streptomycin also had a stimulating effect, whereas chloromycetin was inhibitory.

Jurtshuk, Doetsch, McNeill & Shaw (110) found no evidence for the presence of an enzyme able to inactivate aureomycin or terramycin either in the rumen fluid or in mixed suspensions of rumen organisms. Where a non-specific type of inactivation occurred it seemed probable that it was due to the protein nature of the mixed bacterial suspensions. The presence of both aureomycin and terramycin decreased the ability of rumen bacteria in the resting state to utilize xylose, arabinose, glucose, maltose and cellobiose *in vitro*.

Lodge, Miles & Jacobson (111) have found that while addition of aureomycin to a fermentation mixture in the artificial rumen severely inhibited cellulose digestion the same addition had little effect on the digestion of cellulose by inocula from cows which had already received the antibiotic, though micro-organisms from these animals only digested 58% cellulose compared with 78% for the bacteria from untreated cows. Kesler (112) and Huhtanen, Saunders & Gall (113) have found that terramycin lowered cellulose digestion. The latter authors have observed that aureomycin, penicillin and bacitracin have a similar effect. This result with penicillin contrasts with the stimulatory effect reported by Wasserman *et al.* (109), which is also at variance with the finding of Hardie, Downing, Luther & Reynolds (114) that penicillin-G showed marked inhibition of cellulose digestion. These last authors have studied the effects of various compounds on cellulose digestion *in vitro*, and their results may be summarized as follows: polymyxin-B and chloramphenicol had little effect on cellulolytic activity of rumen micro-organisms; bacitracin and dihydrostreptomycin had a slight effect which increased with increases in the concentration of the antibiotics; oxytetracycline-HCl and chlortetracycline-HCl showed a moderate inhibitory effect which also increased with concentration; carbomycin and penicillin G showed marked inhibition. For a very recent review on the general subject of antibiotics in the nutrition of ruminants the reader is referred to Reid, Warner & Loosli (115).

Of the biochemistry of cellulose breakdown by rumen bacteria little has been established. It seems likely that from the hexose stage the degradation proceeds via the Embden-Meyerhof-Parnas system which has already been discussed (p. 233), but no proof of this has yet been presented. Moreover, some doubt still exists about the identity of the product formed in the initial cleavage reaction. In 1944, Hungate (116), in a study of cellulose fermentation by *Clostridium cellobioparus* isolated from the rumen, was able to show that cellobiose was an important product of the fermentation, but could not detect any glucose. Kitts & Leland (117), on the other hand, stated in 1954 that glucose was the principal intermediate compound in the breakdown of cellulose by rumen organisms. Their evidence was based on filter-paper chromatograms. Clearly, further work is required both with individual species and with samples of the mixed flora of the rumen.

OTHER CARBOHYDRATES

Xylose. The fact that xylose is fermented by mixed bacteria from the rumen of sheep fed on hay to give acetic and propionic acids, with smaller amounts of butyric and formic acids, has been shown by Heald (118). The formation of lactic acid, acetoin and 2:3-butanediol from glucose and cellobiose, but not from xylose or glucuronic acid, by five strains of coliform organisms from the rumen was also shown. The same author (119) has shown that when hay was fed the xylan it

contained was fermented to the extent of 23–26 % in one sheep and 48–52 % in another. The results suggested that 60–80 g. xylan might be fermented in the rumen of a sheep at pasture in 24 hr.

Starch. The passage of starch through the stomach of the sheep has been investigated by Weller & Gray (120). They observed that most of the starch was present in the fluid portion of the contents but that much of it might be carried by the ciliate protozoa. Extensive decomposition of starch appeared to occur in the omasum. When 20–40 g. starch were fed only about one-tenth of that amount appeared to reach the abomasum.

THE VOLATILE FATTY ACIDS

The fact that a large proportion of the carbohydrate ingested by ruminants is converted to acetic, priopionic and butyric acids and that those products are absorbed into the blood from the rumen and reticulum was proved more than 10 years ago by Barcroft, Phillipson & McAnally (121, 122). In ruminants the level of blood glucose is much lower than in non-ruminants, and this is undoubtedly related to the fact that in the ruminant, acetic acid acts as one of the main metabolites. Thus Jarret & Potter (123) found that in the lamb at birth the reducing sugar was 110 mg./100 ml. of which 60 mg./100 ml. were fructose and that the latter fell to 4–8 mg./100 ml. in 5 hr. When the lamb had suckled, the glucose rose and remained at 120 mg./100 ml. for 2 weeks before gradually falling to the adult level of about 50 mg./100 ml. In a brief abstract published in 1954, Conrad, Hibbs & Pounden (124) refer to the influence of the ration on rumen development in the calf. They found that if either alfalfa or clover-timothy hay and grain were fed, with different proportions of hay to grain, the volatile fatty acid content of rumen juice increased from an average of 60 m.equiv./l. at 4 weeks of age to 92 m.equiv./l. at 9 weeks, whereas the blood-sugar level declined from 80–100 mg./ml. to 45–65 mg./100 ml. during the first 8 weeks. As opposed to this the blood-sugar level of calves fed all milk and no roughage and which were thus not ruminating remained at 70–85 mg. glucose/100 ml. blood. The development of the rumen in calves has been discussed by Blaxter (125) in a chapter on ruminant nutrition in a book entitled *Progress in the Physiology of Farm Animals*, edited by Hammond and published in 1954.

By detailed chromatographic analysis Gray, Pilgrim, Rodda & Weller (126) have shown the presence in the rumen of the sheep of formic, acetic, propionic, butyric, *iso*butyric, valeric and caproic acids with possibly a trace of heptoic acid. These authors studied the fermentation of wheat hay in the presence of $CH_3{}^{14}COOH$ and concluded that acetic acid contributed to the formation of valeric and caproic acids. By the same method they came to the conclusion that propionic acid contributed to the formation of valeric acid. El-Shazly (127, 128) has made a very intensive study of the volatile fatty acids in the rumen of the sheep fed on a number of different diets. On a molecular basis acetic acid ranged from 53 to 73 % of the mixed volatile fatty acids, propionic from 14 to 26 %, *n*-butyric from 7 to 19 %, *iso*butyric from 0·5 to 2·8 %, *n*-valeric from 0·6 to 3·3 %, branched C_5 acids from 0·8 to 5·0 % with traces of C_6 and higher acids. The amounts of the branched-chain acids were always higher when casein was present in the diet and when ammonia production was also very marked. El-Shazly concluded that the amino-acids of the protein fed may be the principal source of both the ammonia and the branched-chain acids. This he confirmed by further work with both sheep rumen contents and washed suspensions of rumen bacteria, using also the amino-acids of an acid hydrolysate of casein. In the presence of other amino-acids, L-proline was reduced to give δ-aminovaleric acid which was isolated and characterized. It seems almost certain that the branched-chain acids arise by Stickland reactions (129, 130) from valine, leucine and *iso*leucine. The work of El-Shazly has been continued and extended by Annison (131), who has confirmed the presence of *iso*butyric, *iso*valeric and D-2-methylbutyric acids in the rumen of sheep maintained on various diets. These findings are of great importance in view of the experiments reported in the section on cellulose digestion (p. 239) in which it has been suggested that some of the so-called 'cellulolytic factors' may be combinations of branched-chain acids with straight-chain acids.

The presence of these branched-chain acids in the rumen may partly explain the presence of higher odd-numbered and branched-chain fatty acids in the milk and body fats of ruminants,

which has been shown in recent years by Hansen & Shorland (see, for example, reference nos. 132 and 133), since it is probable that small amounts of the branched-chain compounds can readily be incorporated in fatty acid molecules during the normal synthesis of fatty acids from the usual 2-carbon units.

Although it has been established beyond doubt that the volatile fatty acids form the main hydrolytic products of carbohydrate digestion by rumen bacteria, the reactions by which these acids arise have not yet been fully worked out. As has already been stated (p. 233), it may be that the degradation of carbohydrate passes through glucose and fructose-phosphates and phosphoglyceric acid to pyruvic acid, and it is at this stage that it seems most likely that fatty acids would begin to be formed. Acetic acid and propionic acid seem to account for about 80% of the volatile fatty acid of the rumen. They could be formed via pyruvic and lactic acids, and as has just been indicated, they might arise together with the branched-chain acids from amino-acids by a Stickland type of reaction. Another source of acetic and butyric acids is indicated by the work of Dohner & Cardon (134), who fermented lysine with two strains of *Escherichia coli* isolated from bovine rumen. The nature of the end-products suggested that the reaction was according to the following equation:

$$\underset{\text{L-lysine}}{CH_2NH_2.CH_2CH_2CH_2CH.NH_2COOH} + 2HOH \rightarrow \underset{\text{acetic acid}}{CH_3COOH} + \underset{\text{butyric acid}}{CH_3CH_2CH_2COOH} + 2NH_3$$

LACTIC AND SUCCINIC ACIDS

It is of interest to discuss here the place of succinic acid and of lactic acid in the biochemistry of the rumen. Sijpesteijn (135) has obtained pure cultures of strains of *Ruminococcus flavefaciens* from sheep and bovine rumen. These bacteria are anaerobic Gram-positive organisms which ferment cellulose and cellobiose but not starch, maltose, lactose or xylose. It was shown that at least 25% of the carbon of cellulose or cellobiose decomposed could be recovered as succinic acid and about 23% as acetic and 10% as formic. The quantitative decarboxylation of succinic acid to give propionic acid and CO_2 has been demonstrated by Sijpesteijn & Elsden (136) with washed suspensions of rumen bacteria. The fact that succinic acid can be converted to propionic acid in this way has been studied by Johns (39), who showed that *Veillonella gazogenes* isolated from the rumen of the sheep failed to ferment sugars but could ferment lactate, and suggested (20) that the reaction passes through the following steps:

$$\text{lactate} \rightarrow \text{pyruvate} \xrightarrow{CO_2} \text{oxaloacetate} \rightarrow \text{malate} \rightarrow \text{fumarate} \rightarrow \text{propionate} + CO_2.$$

Doetsch *et al.* (22, 28) have confirmed that succinic acid is degraded by rumen bacteria to give propionic acid, but they found that although fumarate and malate both gave considerable amounts of CO_2, only malate gave propionic acid, fumarate giving rise to acetic acid only. It was shown also that while catabolism of cellobiose, glucose, succinate, fumarate and malate yielded only CO_2, maltose, pyruvate, formate and, to a lesser extent, lactate gave rise to very significant amounts of other unidentified gases. It would appear therefore that several different metabolic pathways may be involved. However true this may be it is clear that succinic acid is an important intermediary in the metabolism of rumen bacteria. Not only can it arise from carbohydrates and lactate, but just recently it has been shown by Sirotnak *et al.* (21) that it can be produced from aspartic acid by mixed suspensions of rumen bacteria, the succinate then giving rise to propionic acid which in turn may be partially converted to acetic and butyric acids.

The effect of the nature and concentration of the substrate, the length of the incubation period, the pH and the presence of chelating agents on lactic acid production by mixed suspensions of bovine rumen bacteria has been studied by Doetsch & Robinson (137), but so far the work has been reported only in a brief abstract which does not give details.

SYNTHESIS OF FATTY ACIDS FROM PYRUVATE

In the study of a Gram-negative coccus isolated from the rumen of the sheep, Elsden & Lewis (138) have shown that this bacterium will attack glucose, fructose, sorbitol, mannitol, lactate, pyruvate, DL-serine and formate anaerobically with the production of CO_2 and a gas which was assumed to be hydrogen. When pyruvate was fermented under an atmosphere of hydrogen there was an initial production of hydrogen followed by a consumption of the gas. Uptake of hydrogen was increased by addition of acetate, propionate or *n*-butyrate to the pyruvate fermentations and at the same time increased formation of *n*-butyrate, *n*-pentanoate and *n*-hexanoate was observed. The authors suggested that the following reactions might be involved where RSH represents coenzyme A:

$$CH_3.C{:}O.COOH + RSH \rightarrow (CH_3CO)SR + 2H^+ + 2e + CO_2,$$

$$2H^+ + 2e \rightarrow H_2,$$

$$CH_3.C{:}O.COOH + 4H^+ + 4e \rightarrow CH_3CH_2COOH + H_2O,$$

$$CH_3.C{:}O.SR + 4H^+ + 4e + \left\{\begin{matrix} CH_3COOH \rightarrow CH_3(CH_2)_2COOH \\ CH_3CH_2COOH \rightarrow CH_3(CH_2)_3COOH \\ CH_3(CH_2)_2COOH \rightarrow CH_3(CH_2)_4COOH \end{matrix}\right\} + RSH + H_2O.$$

The presence of this particular coccus in the rumen might therefore account for the occurrence of volatile acids with five and six carbon atoms in the rumen contents.

THE METABOLISM OF NON-PROTEIN NITROGEN

The fate of nitrogenous compounds reaching the rumen in the food has received much attention of late. Pearson & Smith (139) showed that both synthesis and degradation of protein took place during incubation of rumen contents *in vitro*, the predominance of one over the other appearing to depend on the conditions pertaining at the time. Not very much is yet known about the metabolism of protein in the rumen, but much work has been done in recent years on amino-acid metabolism. Thus in 1952 Bouckaert & Oyaert (140) compared the amino-acid contents of feeds and of rumen fluid before and after cattle were given the feeds. There was a decrease in arginine and histidine but no measurable change in phenylalanine, threonine and valine, while with *iso*leucine, leucine, lysine and methionine an increase occurred. As already mentioned (p. 242), El-Shazly (127, 128) has studied the production of volatile fatty acids from amino-acids. He observed that the decomposition of amino-acids to ammonia and other products by rumen bacteria was greatly increased by the amount of soluble protein in the medium. Sirotnak *et al.* (141, 142) have studied amino-acid breakdown by mixed suspensions of bovine rumen bacteria. The production of CO_2, volatile fatty acids, lactic acid, and total keto acids was determined. The optimum pH for amino-acid breakdown appeared to be 6·9, and the process was markedly enhanced in the presence of readily fermentable carbohydrates such as maltose and cellobiose. Of twenty-two amino-acids tested, only aspartic acid, glutamic acid, serine, arginine, cysteine and cystine were found to be attacked by the bacterial suspensions used. The principal products formed were CO_2, NH_3 and acetic, propionic and butyric acids. Keto acids and lactic acid were not detected. As already mentioned (p. 233) Sirotnak *et al.* (21) have studied the decomposition of aspartic acid by rumen bacteria, and have shown that the principal pathway appears to be deamination to succinic acid followed by decarboxylation to propionic acid.

There is very good evidence that in addition to the acids mentioned above, lysine can be degraded by rumen bacteria. The work of Dohner & Cardon (134) on this subject, in which they showed that lysine could be decomposed to giv eacetic acid, propionic acid and NH_3, has already been discussed (p. 243). Fauconneau & Chevillard (143) incubated rumen liquid from sheep with hay or grass, and concluded that there was a partial destruction of the lysine and cystine of the feedingstuff and possibly a small increase in the total methionine of the mixture.

The utilization of urea continues to be studied, particularly in the United States, but most of

the work consists of feeding experiments in which urea supplies a varying proportion of the nitrogen of the diet. While experiments of that type can give valuable information about urea as a dietary constituent, they naturally do not help much in elucidating the biochemical processes by which non-protein nitrogen is used. Such experiments have been done in recent years by Gallup, Pope & Whitehair (144) with lambs and by Thompson, Graf, Eheart & Holdaway (145) with cattle. In the latter work dairy cows were used, and urea rations in which urea supplied about 29% of the total nitrogen were compared with rations in which cottonseed meal supplied the same proportion of the nitrogen. At the levels used and under the conditions of this experiment (two very important qualifications where urea-feeding is concerned), the inclusion of urea in the rations instead of cottonseed meal did not adversely affect the digestibility of the ration or give any significant difference in milk production. The addition to the urea rations of 22 g. DL-methionine per cow per day did not have any beneficial effect. In growth studies lasting more than two years and involving eighty dairy heifers and eight bull calves, Bohman, Trinberger, Loosli & Turk (146) fed the animals as much low quality hay as they would eat and tested the effect of supplementing this ration with (1) molasses and urea, (2) maize and soybean oil meal, (3) molasses, maize and urea. In every trial but one the animals receiving the soybean oil meal rations with either maize or molasses gained significantly more than the animals receiving urea. The authors attributed the poor response to urea to the inadequacy of molasses to act as a source of readily available carbohydrate for the rumen bacteria. In the past year Belasco (147) has concluded from *in vitro* work that many other substances can act as a source of non-protein nitrogen for the rumen bacteria. The ammonium salts of formic, α-keto-glutaric, malic and especially succinic and lactic acids, gave higher rates of nitrogen utilization and lower free ammonia levels than urea and certain other ammonium salts. Guanidine carbonate, hydrochloride and acetate, when used as the sole added nitrogen source *in vitro*, gave low free ammonia levels and a high level of cellulose digestion. Creatine and creatinine were also effective. The amides of several monocarboxylic acids produced low levels of ammonia, and in providing non-protein nitrogen they stimulated cellulose digestion but perhaps to a smaller degree than urea. The diamides of certain dicarboxylic acids did not appear to be useful in these respects, but uric acid and allantoin were able to provide the bacteria with nitrogen. Further experiments by Belasco (148) indicated that urea was more effective in stimulating cellulose digestion in the artificial rumen under the particular conditions used, than soybean, linseed, bruised cottonseed and maize gluten meals, and that in presence of urea as sole nitrogen source cellulose digestion was stimulated. Stallcup (149) found that urea gave rise to much more ammonia in the artificial rumen than one or two other substances which he tested such as ammoniated molasses and soybean oil meal. Hudman, Kunkel & Hood (150) found that maximum synthesis of protein *in vitro* occurred with urea and ammonium acetate when the rumen inoculum contained 3 g. glucose and 9–15 g. cellulose per litre.

A few years ago McDonald (151) fed to a sheep a diet in which 82% of the protein consisted of zein, and collected samples of the duodenal ingesta from a pyloric fistula. Zein contains no lysine and is soluble in alcohol. From the nature of the duodenal ingesta McDonald calculated that some 40% of the zein in the diet was converted to microbial protein which of course contains lysine. McNaught (152) has also demonstrated the formation of lysine by rumen bacteria *in vitro*. Loosli *et al.* (66) have studied the synthesis of amino-acids in the rumen of sheep and goats by feeding a synthetic diet in which urea supplied almost all the nitrogen and analysing samples of the rumen contents which were obtained by stomach tube. Considerable synthesis occurred of all the amino-acids tested for, the increase in lysine being particularly high. Duncan *et al.* (65) made seven trials in which they fed three rumen-fistulated calves with rations in which urea formed practically the sole source of nitrogen. They analysed the rumen ingesta before and 6 hr. after the calves were fed, and concluded that the amino-acids that are essential for the rat can readily be synthesized in the rumen.

The utilization of sulphur in the synthesis of methionine and cystine has been studied by Block, Stekol & Loosli (153) who found that on feeding $Na_2{}^{35}SO_4$ to a goat the milk proteins showed considerable radioactivity within 3 hr. and showed maximum radioactivity 24 hr. after the feeding.

The activities of methionine and cystine were equal. When the radioactive sulphate was fed to a ewe and the rumen contents sampled from time to time, the greater proportion of the activity was in the protein fraction and again the ratio of the activity in the cystine was equal to that in the methionine. Hale & Garrigus (154) have demonstrated the synthesis of labelled cystine from ingested labelled sulphur by its appearance in wool, and have suggested that sulphur given as the sulphate is more efficiently utilized than elemental sulphur. In the work of Loosli *et al.* (66) and Duncan *et al.* (65), already cited, the synthesis of methionine has been shown to occur in the rumen. It is probable that an important step in the synthesis is the reduction of sulphate to sulphide which Lewis (31) has shown to occur in the rumen of sheep and in washed suspensions of rumen bacteria *in vitro*. Sulphite and thiosulphate were similarly reduced. The possibility of producing toxic levels of hydrogen sulphide by feeding large amounts of sodium sulphate was investigated, but even when 150 g. $Na_2SO_4 . 10H_2O$ were given to a sheep and the sulphide in the rumen liquid reached a concentration of 14·7 μmoles/ml. no ill effects were observed. The incorporation of sulphide into the sulphur-containing amino-acids is probably rapid.

Several attempts have been made to determine how much protein is synthesized in the rumen. Agrawala, Duncan & Huffman (155) calculate from the results of experiments with calves of over 6 months old that the amount varied from 33 to 109 g. in 6 hr. From the results of *in vitro* incubations obtained by Pearson & Smith (139), McNaught & Smith (3) calculated that in the adult bovine 100–150 g. protein might be formed per day. Gray, Pilgrim & Weller (156) have studied the extent to which the plant protein of the feed was converted to microbial protein in the rumen of the sheep, and concluded that when wheaten hay was fed there was a conversion of about half the plant nitrogen to microbial nitrogen. Reference has already been made to the work of McDonald (151), in which he concluded that with zein as the main protein of the diet 40% of it was converted to microbial protein. In a more recent paper (157) he suggests that zein is too glutinous to be well utilized by the micro-organisms, and that the figure of 40% would probably be higher with other dietary proteins.

VITAMINS

Early work on the synthesis of vitamins by the micro-organisms of the rumen was very thoroughly reviewed by Kon & Porter (3) in 1947. The synthesis of vitamins, particularly of the B complex, has been studied by many workers, but in this review consideration is given only to those papers which have been published since the end of 1951.

Kesler & Knodt (158) have determined the amounts of various B vitamins in the contents of different parts of the alimentary tracts of young calves. They found that the concentrations of thiamine, riboflavin, niacin and pteroylglutamic acid were higher in the contents of the rumen, abomasum, small intestine and large intestine than in the feed consumed. Conrad, Hibbs & Pounden (159) also using calves fed various types of roughage diet found that a sharp increase in both riboflavin and thiamine occurred as soon as the calf started to eat hay, the rumen level of the vitamin being higher in calves that were inoculated with rumen liquid from other animals than in the rumen contents of their uninoculated pair mates. Rumen-fistulated calves were used in seven trials by Agrawala, Huffman, Leucke & Duncan (160) to study the synthesis of riboflavin, pantothenic acid and niacin when a purified ration was fed in which urea supplied the only significant source of nitrogen. Evidence was obtained that the rumen micro-organisms were able to synthesize as much riboflavin and niacin in 6 hr. as was found in the rumen of a calf maintained on a normal ration, but only 50% as much pantothenic acid. The actual amounts of riboflavin, pantothenic acid and niacin calculated to be synthesized in 6 hr. were 20, 31 and 154 mg. respectively. Hollis, Chappel, MacVicar & Whitehair (161) have observed increased production of these three vitamins when either soybean meal or urea was added to a basal diet of hay. They have found also that addition of alfalfa ash to a ratio containing corn cobs as the principal source of roughage, resulted in increased synthesis of all three vitamins.

A study has been made by Conrad & Hibbs (162) of the effect of feeding diets containing different proportions of hay to grain with different types of hay, on the synthesis of thiamine and riboflavin

in the rumen of young calves which had been rumen-inoculated. They concluded that the synthesis of these vitamins in the rumen was not affected, provided good quality legume or mixed legume hay was fed, but when natural timothy hay formed a substantial proportion of the diet, highly significant differences were observed in the levels of the vitamins in the rumen between groups of calves fed different proportions of hay to grain.

Perhaps the vitamin whose synthesis in the rumen has received most attention in recent years is vitamin B_{12}. Many of those who have worked on this subject were previously interested in cobalt deficiency in animals. Anderson & Andrews (163) showed that therapeutic response of lambs which developed signs of cobalt deficiency under field conditions was the same whether B_{12} was injected or cobalt was given by mouth. Much the same was found by Marston & Lee (164) and Marston & Smith (165), who pointed out that the requirement of the ruminant for vitamin B_{12} was exceptionally high and that when the cobalt content of the diet imposed no limits, the amount of vitamin B_{12} that was synthesized was probably very great. Phillipson & Mitchell (166) obtained confirmatory evidence of this, for in their work cobalt injected intravenously or into the duodenum did not cure the cobalt deficient lamb unless it found its way into the rumen perhaps via the saliva or by ingestion of faeces. It is important to note that lambs in an advanced state of deficiency did not respond to orally administered cobalt, and it was suggested that this might be due to the fact that in advanced deficiency the rumen flora has altered considerably. Tribe, Bond & Osborne (167) found that sheep fed a diet low in cobalt gained weight and ate more food when given 50,000 units of sodium penicillin-G every day for a period of 5 weeks. The changes that took place were similar to those that would have been expected had cobalt or vitamin B_{12} been given, and it is suggested that the penicillin so altered the flora of the rumen that the bacteria now flourishing were able to make better use of the small amounts of cobalt in the diet.

Hartman & Dryden (168), by adding cobalt to the ration and using rats for the assay of vitamin B_{12}, failed to increase the concentration of the vitamin in cows' milk from an initial average of 7·7 μg. per litre. Harper, Richard & Collins (169), in similar experiments but using a microbiological assay, increased the B_{12} concentration in the milk of ewes from an initial level of 1·4 to 9·6 μg per litre. Coates, Ford, Harrison, Kon & Porter (170), in an extensive study of vitamin B_{12}-like compounds, have obtained very strong evidence that there were B_{12}-like compounds present in calf-gut contents and faeces which were not convertible to cyanocobalamin by cyanide and had different activities for chicks and for several test micro-organisms. Differences in response with different test organisms were also observed by Dawbarn, Hine & Hughes (171), who concluded that there are at least two vitamin B_{12}-like factors in sheep faeces, and that their absolute and relative concentrations in the faeces depended upon the level of available cobalt in the gut.

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REFERENCES

(1) Owen (1954). *J. Dairy Res.* **21**, 408.
(2) Krebs (1937). *Biederm. Zbl.* **9**, 394.
(3) Baker, Harriss, Phillipson, McNaught, Smith, Kon & Porter (1947). *Nutr. Abstr. Rev.* **17**, 1.
(4) Phillipson (1946). *Vet. Rec.* **58**, 81.
(5) Elsden & Phillipson (1948). *Annu. Rev. Biochem.* **17**, 705.
(6) Doetsch & Robinson (1953). *J. Dairy Sci.* **36**, 115.
(7) Underwood & Moir (1953). *J. Aust. Inst. agric. Sci.* **19**, 214.
(8) Dale, Stewart & Brody (1954). *Cornell Vet.* **44**, 368.
(9) Ammerman & Thomas (1952). *J. Anim. Sci.* **11**, 754.
(10) Cason, Ruby & Stallcup (1954). *J. Nutr.* **52**, 457.
(11) Markoff (1913). *Biochem. Z.* **57**, 1.
(12) Nasr (1950). *J. agric. Sci.* **40**, 308.

(13) Mangold (1929). *Handbook of Nutrition and Metabolism of Domesticated Animals. 2. Digestion and Excretion.* Berlin: Julius Springer.
(14) McDougal (1948). *Biochem. J.* **43**, 99.
(15) Clark (1953). *Onderstepoort J. vet. Sci.* **26**, 137.
(16) Cole, Huffman, Kleiber, Olson & Schalk (1945). *J. Anim. Sci.* **4**, 183.
(17) Kleiber, Cole & Mead (1943). *J. Dairy Sci.* **26**, 929.
(18) Stephenson (1949). *Bacterial Metabolism*, 3rd ed. London: Longmans.
(19) Nisman (1954). *Bact. Rev.* **18**, 16.
(20) Johns (1951). *J. gen. Microbiol.* **5**, 326.
(21) Sirotnak, Doetsch, Robinson & Shaw (1954). *J. Dairy Sci.* **37**, 531.
(22) Doetsch, Robinson, Brown & Shaw (1953). *J. Dairy Sci.* **36**, 825.
(23) Masson & Phillipson (1951). *J. Physiol.* **113**, 189.
(24) Huhtanen, Carleton & Roberts (1954). *J. Bact.* **68**, 749.
(25) Gall & Huhtanen (1951). *J. Dairy Sci.* **34**, 353.
(26) Huhtanen & Gall (1953). *J. Bact.* **65**, 554.
(27) Beijer (1952). *Nature, Lond.*, **170**, 576.
(28) Doetsch, Robinson & Shaw (1953). *J. Dairy Sci.* **36**, 588.
(29) McNeill & Brown (1954). *J. Dairy Sci.* **37**, 662.
(30) Davis & Squires (1954). *Science*, **119**, 381.
(31) Lewis (1954). *Biochem. J.* **56**, 391.
(32) Rosebury, Gale & Taylor (1954). *J. Bact.* **67**, 135.
(33) Gyllenberg (1953). *Acta agric. Scand.* **2**, 183.
(34) Moir & Masson (1952). *J. Path. Bact.* **64**, 343.
(35) Heald, Krogh, Mann, Appleby, Masson & Oxford (1953). *J. gen. Microbiol.* **9**, 107.
(36) Mann, Masson & Oxford (1954). *J. gen. Microbiol.* **10**, 142.
(37) Higginbottom & Wheater (1954). *J. agric. Sci.* **44**, 434.
(38) Gutierrez (1953). *J. Bact.* **66**, 123.
(39) Johns (1951). *J. gen. Microbiol.* **5**, 317.
(40) Bryant & Burkey (1953). *J. Dairy Sci.* **36**, 205.
(41) Baker (1942). *Nature, Lond.*, **149**, 220.
(42) Baker, Nasr, Morrice & Bruce (1950). *J. Path. Bact.* **62**, 617.
(43) Doetsch, Robinson & Shaw (1952). *J. Anim. Sci.* **11**, 536.
(44) Huhtanen & Gall (1953). *J. Bact.* **65**, 548.
(45) McNeill, Doetsch & Robinson (1953). *J. Dairy Sci.* **36**, 588.
(46) McNeill, Doetsch & Shaw (1954). *J. Dairy Sci.* **37**, 81.
(47) Wasserman, Seeley & Loosli (1953). *J. Anim. Sci.* **12**, 935.
(48) Wasserman, Seeley & Loosli (1954). *J. Dairy Sci.* **37**, 663.
(49) Bentley, Lehmkuhl, Johnson, Hershberger & Moxon (1954). *J. Amer. chem. Soc.* **76**, 5000.
(50) Bryant & Doetsch (1954). *Science*, **120**, 3127.
(51) Wilson & Briggs (1954). *Vet. Rec.* **66**, 187.
(52) Quin, Oyaert & Clark (1951). *Onderstepoort J. vet. Sci.* **25**, 51.
(53) Gall, Thomas, Loosli & Huhtanen (1951). *J. Nutr.* **44**, 113.
(54) Gall, Huhtanen, Saunders & Schmidt (1953). *J. Dairy Sci.* **36**, 587.
(55) Bryant & Burkey (1953). *J. Dairy Sci.* **36**, 218.
(56) Bryant & Burkey (1953). *J. Dairy Sci.* **36**, 588.
(57) Williams, Nottle, Moir & Underwood (1953). *Aust. J. Biol. Sci.* **6**, 142.
(58) Hungate, Dougherty, Bryant & Cello (1952). *Cornell Vet.* **42**, 423.
(59) Scarisbrick (1954). *Vet. Rec.* **66**, 131.
(60) Johansen, Moir & Underwood (1949). *Nature, Lond.*, **163**, 101.
(61) Holmes, Moir & Underwood (1953). *Aust. J. Biol. Sci.* **6**, 637.
(62) Tsuda (1953). *Tohoku J. agric. Res.* **3**, 255.
(63) Synge (1953). *J. gen. Microbiol.* **9**, 407.
(64) Synge (1952). *Proc. roy. Soc.* B, **139**, 205.
(65) Duncan, Agrawala, Huffman & Luecke (1953). *J. Nutr.* **49**, 41.
(66) Loosli, Williams, Thomas, Ferris & Maynard (1949). *Science*, **110**, 144.

(67) Hobson & MacPherson (1953). *Nature, Lond.*, **171**, 129.
(68) Greenwood (1954). *Biochem. J.* **57**, 151.
(69) Davidson (1954). *J. Sci. Fd Agric.* **5**, 86.
(70) Antoniani (1935). *Biochim. Terap. sper.* **22**, 48.
(71) Hobson & MacPherson (1952). *Biochem. J.* **52**, 671.
(72) Karunairatnam & Levvy (1951). *Biochem. J.* **49**, 210.
(73) Marsh (1954). *Biochem. J.* **58**, 609.
(74) Conchie (1954). *Biochem. J.* **58**, 552.
(75) Sym (1938). *Acta Biol. exp., Varsovie*, **12**, 192.
(76) Kimura & Umezu (1954). *Tohoku J. agric. Res.* **4**, 141.
(77) Pearson & Smith (1943). *Biochem. J.* **37**, 148.
(78) Rodwell (1953). *J. gen. Microbiol.* **8**, 224.
(79) Rodwell (1953). *J. gen. Microbiol.* **8**, 233.
(80) Becker & Talbott (1927). *Iowa St. Coll. J. Sci.* **1**, 345.
(81) Sugden & Oxford (1952). *J. gen. Microbiol.* **7**, 145.
(82) Sugden (1953). *J. gen. Microbiol.* **9**, 44.
(83) Oxford (1951). *J. gen. Microbiol.* **5**, 83.
(84) Masson & Oxford (1951). *J. gen. Microbiol.* **5**, 664.
(85) Heald & Oxford (1953). *Biochem. J.* **53**, 506.
(86) Forsyth & Hirst (1953). *J. chem. Soc.* p. 2131.
(87) Forsyth, Hirst & Oxford (1953). *J. chem. Soc.* p. 2030.
(88) Kameoka, Takajasji & Morimoro (1954). *Bull. nat. Inst. agric. Sci.* (Series G, Japan), **8**, 75.
(89) McNaught, Owen, Henry & Kon (1954). *Biochem. J.* **56**, 151.
(90) McNaught, Smith, Henry & Kon (1950). *Biochem. J.* **46**, 32.
(91) Reed, Moir & Underwood (1949). *Aust. J. sci. Res.* B, **2**, 304.
(92) D'Agostino (1951). *Boll. Soc. ital. Biol. sper.* **27**, 1551.
(93) MacLeod & Brumwell (1954). *Appl. Microbiol.* **2**, 130.
(94) Cardon (1953). *J. Anim. Sci.* **12**, 536.
(95) Burroughs, Latona, DePaul, Gerlaough & Bethke (1951). *J. Anim. Sci.* **10**, 693.
(96) Brooks, Garner, Gehrke, Muhrer & Pfander (1954). *J. Anim. Sci.* **13**, 758.
(97) Brooks, Garner, Muhrer & Pfander (1954). *Science*, **120**, 455.
(98) Garner, Muhrer, Ellis & Pfander (1954). *Science*, **120**, 435.
(99) Hall, Cheng & Burroughs (1953). *J. Anim. Sci.* **12**, 918.
(100) Ruf, Hale & Burroughs (1953). *J. Anim. Sci.* **12**, 731.
(101) Bentley, Johnson, Vanecko & Hunt (1954). *J. Anim. Sci.* **13**, 581.
(102) Bentley, Johnson & Moxon (1952). *Fed. Proc.* **13**, 182.
(103) Hunt, Bentley, Hershberger & Cline (1954). *J. Anim. Sci.* **13**, 570.
(104) Oyaert, Quin & Clark (1951). *Onderstepoort J. vet. Sci.* **25**, 59.
(105) Turner & Hodgetts (1952). *Aust. J. agric. Res.* **3**, 453.
(106) Chance, Huffman & Duncan (1953). *J. Dairy Sci.* **36**, 253.
(107) Chance, Duncan, Huffman & Leucke (1953). *J. Dairy Sci.* **36**, 495.
(108) Chance, Smith, Huffman & Duncan (1953). *J. Dairy Sci.* **36**, 743.
(109) Wasserman, Duncan, Churchill & Huffman (1952). *J. Dairy Sci.* **35**, 571.
(110) Jurtshuk, Doetsch, McNeill & Shaw (1954). *J. Dairy Sci.* **37**, 1466.
(111) Lodge, Miles & Jacobson (1954). *J. Dairy Sci.* **37**, 663.
(112) Kesler (1954). *J. Anim. Sci.* **13**, 10.
(113) Huhtanen, Saunders & Gall (1954). *J. Dairy Sci.* **37**, 328.
(114) Hardie, Downing, Luther & Reynolds (1953–4). *Antibiotics Ann.* (1953). *Proc. Symposium Antibiotics* (*Wash. D.C.*), pp. 372–9.
(115) Reid, Warner & Loosli (1954). *J. agric. Fd Chem.* **2**, 186.
(116) Hungate (1944). *J. Bact.* **48**, 499.
(117) Kitts & Leland (1954). *J. agric. Fd Chem.* **2**, 639.
(118) Heald (1952). *Biochem. J.* **50**, 503.
(119) Heald (1953). *Brit. J. Nutr.* **7**, 124.
(120) Weller & Gray (1954). *J. exp. Biol.* **31**, 40.

(121) Phillipson & McAnally (1942). *J. exp. Biol.* **19**, 199.
(122) Barcroft, McAnally & Phillipson (1944). *J. exp. Biol.* **20**, 120.
(123) Jarret & Potter (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 207.
(124) Conrad, Hibbs & Pounden (1954). *J. Dairy Sci.* **37**, 664.
(125) Blaxter (1954). 'Ruminant Nutrition'. Chapter in *Progress in the Physiology of Farm Animals*, vol. **1**, p. 3, edited by J. Hammond. London: Butterworths Press.
(126) Gray, Pilgrim, Rodda & Weller (1951). *Nature, Lond.*, **167**, 954.
(127) El-Shazly (1952). *Biochem. J.* **51**, 640.
(128) El-Shazly (1952). *Biochem. J.* **51**, 647.
(129) Stickland (1934). *Biochem. J.* **28**, 1746.
(130) Stickland (1935). *Biochem. J.* **29**, 288.
(131) Annison (1954). *Biochem. J.* **57**, 400.
(132) Hansen & Shorland (1951). *Biochem. J.* **50**, 207.
(133) Hansen, Shorland & Cooke (1954). *Biochem. J.* **58**, 358.
(134) Dohner & Cardon (1954). *J. Bact.* **67**, 608.
(135) Sijpesteijn (1951). *J. gen. Microbiol.* **5**, 869.
(136) Sijpesteijn & Elsden (1952). *Biochem. J.* **52**, 41.
(137) Doetsch & Robinson (1954). *J. Dairy Sci.* **37**, 662.
(138) Elsden & Lewis (1953). *Biochem. J.* **55**, 183.
(139) Pearson & Smith (1943). *Biochem. J.* **37**, 153.
(140) Bouckaert & Oyaert (1952). *Zootechnia*, **1**, no. 6, 21.
(141) Sirotnak & Brown (1953). *J. Dairy Sci.* **36**, 588.
(142) Sirotnak, Doetsch, Brown & Shaw (1953). *J. Dairy Sci.* **36**, 1117.
(143) Fauconneau & Chevillard (1953). *C.R. Soc. biol., Paris*, **147**, 69.
(144) Gallup, Pope & Whitehair (1952). *J. Anim. Sci.* **11**, 621.
(145) Thomson, Graf, Eheart & Holdaway (1952). *J. Dairy Sci.* **35**, 1010.
(146) Bohman, Trinberger, Loosli & Turk (1954). *J. Dairy Sci.* **37**, 284.
(147) Belasco (1954). *J. Anim. Sci.* **13**, 601.
(148) Belasco (1954). *J. Anim. Sci.* **13**, 739.
(149) Stallcup (1954). *J. Dairy Sci.* **37**, 1449.
(150) Hudman, Kunkel & Hood (1953). *J. agric. Fd Chem.* **1**, 1060.
(151) McDonald (1948). *J. Physiol.* **107**, 21*P*.
(152) McNaught (1951). *Biochem. J.* **50**, i.
(153) Block, Stekol & Loosli (1951). *Arch. Biochem.* **33**, 353.
(154) Hale & Garrigus (1953). *J. Anim. Sci.* **12**, 492.
(155) Agrawala, Duncan & Huffman (1953). *J. Nutr.* **49**, 29.
(156) Gray, Pilgrim & Weller (1953). *Nature, Lond.*, **172**, 347.
(157) McDonald (1954). *Biochem. J.* **56**, 120.
(158) Kesler & Knodt (1952). *Proc. Soc. exp. Biol., N.Y.*, **79**, 85.
(159) Conrad, Hibbs & Pounden (1952). *J. Anim. Sci.* **11**, 759.
(160) Agrawala, Huffman, Leucke & Duncan (1953). *J. Nutr.* **49**, 631.
(161) Hollis, Chappel, MacVicar & Whitehair (1954). *J. Anim. Sci.* **13**, 732.
(162) Conrad & Hibbs (1954). *J. Dairy Sci.* **37**, 512.
(163) Anderson & Andrews (1952). *Nature, Lond.*, **170**, 807.
(164) Marston & Lee (1952). *Nature, Lond.*, **170**, 791.
(165) Marston & Smith (1952). *Nature, Lond.*, **170**, 792.
(166) Phillipson & Mitchell (1952). *Brit. J. Nutr.* **6**, 176.
(167) Tribe, Bond & Osborne (1954). *Nature, Lond.*, **173**, 728.
(168) Hartman & Dryden (1952). *Arch. Biochem.* **40**, 310.
(169) Harper, Richard & Collins (1951). *Arch. Biochem.* **31**, 328.
(170) Coates, Ford, Harrison, Kon & Porter (1953). *Brit. J. Nutr.* **7**, 319.
(171) Dawbarn, Hine & Hughes (1952). *Nature, Lond.*, **170**, 793.

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